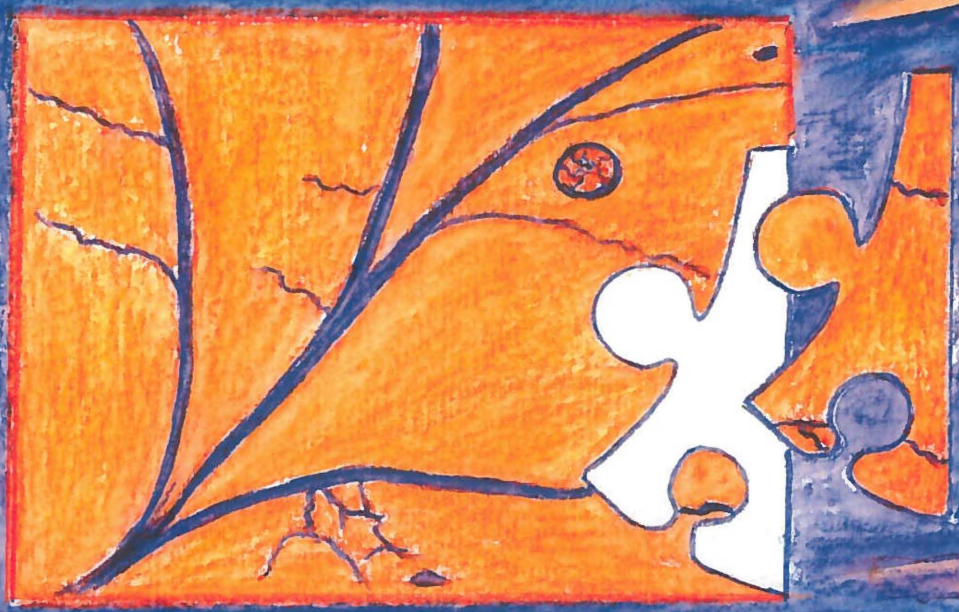
Early clinical studies to explore candidate biomarkers in targeted cancer therapies

Oldenhuis, Corina Neeltje Anna Maria



Early clinical studies to explore candidate biomarkers in targeted cancer therapies

Corina Oldenhuis

Early clinical studies to explore candidate biomarkers in targeted cancer therapies

Corina Oldenhuis

Stellingen behorende bij het proefschrift
'Early clinical studies to explore candidate biomarkers in targeted cancer therapies'

1. Mapatumumab kan zonder duidelijke toename van toxiciteit gecombineerd worden met gemcitabine en cisplatin-bevattende chemotherapie (dit proefschrift).

2. Het toevoegen van de VEGF-receptor tyrosine kinase remmer tivozanib aan de combinatie van FOLFOX (fluorouracil, oxaliplatin en leucovorin) geeft niet meer of ernstiger bijwerkingen dan FOLFOX of tivozanib alleen (dit proefschrift).

3. Het combineren van tivozanib met oxaliplatin en fluorouracil geeft geen farmacokinetische interacties tussen deze middelen (dit proefschrift).

4. Het is mogelijk tumorlaesies met ^{111}In-mapatumumab scintigrafie in beeld te brengen; de eventuele waarde hiervan voor de patiënt moet nader worden vastgesteld (dit proefschrift).

5. Dezelfde biomarker kan zowel prognostisch als predictief zijn (dit proefschrift).

6. Het intranasale Norwalk virus-like particle heeft een beschermende werking tegen de ontwikkeling van norovirus gastroenteritis en vaccinatie dient daarom te worden overwogen in de populatie met een hoog risico (Atmar et al, N Engl J Med 2011).

7. Dat gletsjers veel langzamer smelten dan tot recent werd gedacht, betekent nog niet dat de achteruitgang in omvang niet dramatisch is (o.a. Jacob *et al*, Nature 2012).

8. Gedurende een economische crisis wordt juist het onbetaalbare steeds waardevoller.

9. Het is per definitie onmogelijk om aan te tonen dat iets niet kan (M. Bulnes).

10. Bij homeopathische middelen is het geloof erin het enige werkzame bestanddeel (M. Houben).

11. De eerste regel voor klussers 'bewaar alle onderdelen', is ook zeker van toepassing op het schrijven van een proefschrift (S. IJzer).

12. Voor wie wacht komt alles steeds te laat (Thé Lau).

Corina Oldenhuis, 18 april 2012

Early clinical studies to explore candidate biomarkers in targeted cancer therapies

Oldenhuis, C.N.A.M.

Printing of this thesis was financially supported by: University of Groningen, University Medical Center Groningen, Stichting Werkgroep Interne Oncologie.

Novartis Oncology,
Boehringer Ingelheim BV
Amgen BV, Bayer BV, Takeda

Cover design: Gré Grummel-Wittendorp

Layout: Corina Oldenhuis

Printed by: Ipskamp Drukkers, Enschede, the Netherlands

CMB

rijksuniversiteit groningen

Early clinical studies to explore candidate biomarkers in targeted cancer therapies

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 18 april 2012
om 12.45 uur

door

Corina Neeltje Anna Maria Oldenhuis

geboren op 24 juli 1979
te Emmen

Promotores:	Prof. dr. J.A. Gietema
	Prof. dr. E.G.E. de Vries
	Prof. dr. S. de Jong
Beoordelingscommissie:	Prof. dr. J.G.W. Kosterink
	Prof. dr. S. Sleijfer
	Prof. dr. F.A.E. Kruyt

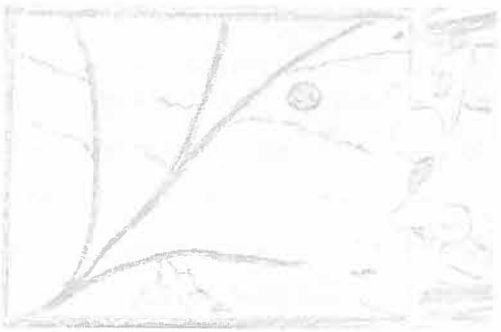
CONTENTS

Chapter 1	Introduction	9
Chapter 2	Targeting TRAIL death receptors	15
Chapter 3	Mapatumumab, a fully human agonistic monoclonal antibody that targets TRAIL-R1, in combination with gemcitabine and cisplatin: a phase I study	31
Chapter 4	A phase Ib study of the VEGF receptor tyrosine kinase inhibitor tivozanib and FOLFOX6 in patients with advanced gastrointestinal malignancies: a preliminary thesis report	53
Chapter 5	Prognostic versus predictive value of biomarkers in oncology	69
Chapter 6	Sulindac inhibits β-catenin expression in normal-appearing colon of Hereditary Nonpolyposis Colorectal Cancer and Familial Adenomatous Polyposis patients	89
Chapter 7	Serial ^{111}In-mapatumumab SPECT scan in cancer patients treated with mapatumumab, gemcitabine and cisplatin.	103
Chapter 8	Single nucleotide polymorphism A683C in TRAIL-receptor 1 and disease outcome after mapatumumab: an exploratory study	121
Chapter 9	Summary, general discussion and future perspectives	133
Chapter 10	Samenvatting, algemene discussie en toekomstperspectieven	147
	Dankwoord	163

Paranimfen: Rian Busstra

Geanne Thole

CHAPTER 1



Introduction

Background

In the past four decades, the mainstay of anticancer treatment has been surgery, radiotherapy, and systemic therapy comprising antihormonal treatment, immunotherapy, targeted agents and chemotherapy. Despite improvement in these treatment modalities, still half of the patients die as a consequence of metastatic disease. For patients with metastatic solid tumors in general only systemic therapy remains an option. However, a major problem of systemic therapy is that the tumor often develops resistance for the applied drugs.

Chemotherapy-resistant tumor cells, for example, are characterized by an intrinsic or acquired incapability of going into apoptosis as a consequence of defects in the intrinsic apoptotic pathway. One strategy to overcome this resistance might be targeting the extrinsic pathway. This pathway is activated by the endogenously present Tumor Necrosis Factor (TNF) Related Apoptosis-Inducing Ligand (TRAIL, or Apo2L). TRAIL can bind to five different receptors, but induces apoptosis only after binding to its two death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5). The other three receptors, TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and the soluble osteoprotegerin (OPG), act as decoys. The finding that TRAIL only induces apoptosis in several tumor cells and not in normal cells makes it a potentially attractive anti-cancer compound. A recombinant human (rh) form of TRAIL, targeting all TRAIL-Rs, and monoclonal antibodies that only target either TRAIL-R1 or TRAIL-R2 were proven safe as single agent in phase 1 and phase 2 trials. Moreover, numerous preclinical studies showed synergy when combining these TRAIL-receptor targeting agents with chemotherapy. In addition, many chemotherapy-resistant cell lines as well as xenograft models could be sensitized to chemotherapy by the addition of rhTRAIL or one of the TRAIL-R1/TRAIL-R2 antibodies.

Another potential approach to treat malignancies is blocking angiogenesis, the formation of new blood vessels. Tumors require an adequate vascularization for obtaining oxygen and nutrients, thereby stimulating tumor growth and the development of metastases. An important proangiogenic protein is Vascular Endothelial Growth Factor (VEGF), known to be overexpressed by many tumor types.

VEGF binding to VEGF-receptors (VEGFR) present on endothelial cells can be prevented by drugs blocking VEGF directly or by inhibiting the tyrosine kinase activity of the receptors.

TRAIL-R targeting agents and angiogenesis inhibitors are examples of targeted therapies, i.e. drugs acting against a specific tumor cell characteristic. Anticancer drugs, and typically targeted therapies, are beneficial in only a subgroup of patients. Better upfront patient selection before therapy or early selection of patients during treatment is needed to prevent unnecessary toxicity and time loss in non-responders.

The aim of this thesis is to study the safety, tolerability and pharmacokinetics of novel targeted anti-cancer agents and to concurrently develop predictive biomarkers for better upfront and early selection of patients.

Outline of the thesis

In **chapter 2**, an overview of the available literature concerning the TRAIL pathway and its exploitation for use in the clinic is given. The current knowledge on the physiological and pathophysiological role of TRAIL and its receptors is described. Furthermore, preclinical and clinical studies with rhTRAIL and TRAIL-R antibodies are reviewed. In addition, studies on potential powerful combinations with chemotherapy and other targeting agents are highlighted for the chemotherapy-resistant glioblastoma models and the combination of rhTRAIL with bortezomib is presented as an interesting strategy.

Chapter 3 focuses on the results of a phase 1 study combining the TRAIL-R1 antibody mapatumumab with chemotherapy. We administered escalating doses of mapatumumab to patients with advanced solid tumors in combination with standard doses gemcitabine and cisplatin. Toxicity and tolerability were closely monitored. In addition, pharmacokinetic interactions of the drugs were studied and preliminary hints of activity were assessed.

Drugs solely targeting tumor cells are probably not sufficient to optimally treat malignancies. Increasingly, the critical role of the microenvironment in tumor development, migration and metastasis is acknowledged. A crucial role in the

microenvironment is played by angiogenic factors. Tivozanib is an orally available VEGF-R1, VEGF-R2 and VEGF-R3 tyrosine kinase inhibitor that showed potent anticancer activity in vitro and in preclinical in vivo models. Tivozanib appeared to be safe as single agent in advanced solid tumor patients and in preclinical models synergistic effects were observed when combined with chemotherapy. These data have led to the phase 1 study, for which preliminary data are reported in **chapter 4**. In this dose escalation study, tivozanib was combined with the well known regimen for gastrointestinal tumors consisting of fluorouracil, oxaliplatin and leucovorin (FOLFOX6). Eligible were patients with advanced gastrointestinal malignancies. The safety of administering this combination was observed and pharmacokinetic profiles were studied. Additional patients at the highest dose level were enrolled to collect more robust safety, pharmacokinetic and efficacy data.

Targeted therapies have in common that they act against a specific feature of tumor cells. Tumor heterogeneity is commonly present, even in patients with the same tumor type, and the target is not always expressed. It is a major challenge to select patients upfront or early during treatment who will benefit from these treatments, while correctly withholding it from patients who will not. Identification of, preferably easy obtainable, predictive biomarkers might help in this process. This is the focus of the second part of this thesis. First, in **chapter 5**, the confusing terminology of prognostic and predictive biomarkers is clarified. In this review examples of strong prognostic and predictive markers are provided and the progress in current biomarker development is described. Suggestions for future research are done as well.

Of the common solid tumors, tumorigenesis is best understood for colorectal cancer (CRC). Approximately 5-10% of the CRCs are part of the well-characterized hereditary forms hereditary nonpolyposis colorectal cancer (HNPCC, or Lynch syndrome) and familial adenomatous polyposis (FAP). Since most of these patients develop CRC during their life, apart from screening strategies, treatment strategies are sought to prevent tumor development. Chemoprevention with non-steroidal anti-inflammatory drugs (NSAIDs) reduced the number of polyps in FAP patients, where its role in HNPCC is still

under debate. However, the exact mechanisms are incompletely understood. Previous studies indicated that NSAIDs seemed to be involved in apoptosis induction as well as in inhibition of proliferation. The NSAID sulindac showed in earlier studies upregulation of TRAIL-R2 in vitro and downregulation of anti-apoptotic proteins like Bcl-X_L. Furthermore, sulindac was found to be involved in the Wingless-int (Wnt) pathway. The Wnt signaling pathway plays an important role in neoplastic transformation of colonic epithelial cells. Mutations in the adenomatous polyposis coli (*APC)* or *β-catenin* gene lead to accumulation and translocation of β-catenin to the nucleus. This results in activation of T-cell factor 4 and subsequently activation of carcinogenic genes. Moreover, active Wnt-signaling reduces p21 levels, allowing cells to proliferate instead of differentiate. Sulindac metabolites showed to decrease β-catenin expression and to induce p21 expression in colon cancer cells. In addition, sulindac induced p21 expression in a mouse model. To gain further insight in the chemopreventive mechanisms of sulindac, we performed a biomarker study. This study is described in **chapter 6**. Biopsies of normal-appearing colonic mucosa obtained in two already publised studies in FAP and HNPCC patients before and after sulindac treatment were studied for apoptosis and expression of TRAIL-R1, TRAIL-R2, p21 and β-catenin by immunohistochemistry.

It is unknown if the TRAIL-R1 targeting agent mapatumumab actually reaches the tumor cells within a patient's tumor to display its anticancer efficacy. Also the effect of chemotherapy on mapatumumab binding to a patient's tumor is not clear, although upregulation of TRAIL-R1 after chemotherapy was seen in mice bearing human colorectal cancer tumor xenografts. Previously, indium-111 (^{111}In) radiolabeled mapatumumab was developed and prepared for clinical use. The tracer showed specific tumor uptake in high TRAIL-R1 expressing human xenografts in mice. In **chapter 7** we give insight into the tumor uptake, pharmacokinetics and biodistribution of ^{111}In-mapatumumab in patients with advanced solid tumors, treated with gemcitabine, cisplatin and mapatumumab. Scans are performed at start of treatment

and after 3 cycles of therapy, to elucidate also the effect of treatment on mapatumumab tumor uptake.

The extrinsic apoptotic pathway is activated after ligand binding to TRAIL-R1 and/or TRAIL-R2. However, presence of the receptor is not sufficient to predict response to TRAIL-R targeting drugs. Mutations in death receptors or changes in expression of pro- and anti-apoptotic downstream proteins might also play a role in insufficient apoptosis induction. Recently, the single nucleotide polymorphism (SNP) A683C in the *TNFRSF10A* gene, encoding TRAIL-R1, was identified. Cancer cell lines carrying the variant were resistant to TRAIL, in contrast to the wildtype carriers. In **chapter 8** we analyzed the effect of SNP A683C in germline DNA samples of patients treated with gemcitabine, cisplatin and mapatumumab in the two previously performed studies described in chapter 3 and chapter 7. For this purpose we performed an analysis of the TRAIL-R1 polymorphism A683C in relation to disease outcome and toxicity.

Finally, in **chapter 9**, the study results incorporated in this thesis are summarized and suggestions are made for future research.

CHAPTER 2

Targeting TRAIL death receptors

CNAM Oldenhuis, JH Stegehuis, AME Walenkamp, S de Jong, EGE de Vries.

Department of Medical Oncology, University Medical Center Groningen, The Netherlands.

Current Opinion in Pharmacology. 2008;8:433-9.

Abstract

The natural occurring tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis following binding to the two TRAIL death receptors (DRs). Its recombinant form and monoclonal antibodies against the TRAIL DRs induce cell death in a wide variety of tumor cell lines and xenografts without causing toxicity to normal cells and are therefore potential attractive anticancer agents. These agents are currently in early clinical development. The phase 1 and 2 studies showed until now limited toxicity and tumor responses have been observed. Ongoing studies focus especially on combination of these agents with other targeted therapies or cytotoxic therapies. In this review, we summarize current knowledge on these agents and highlight their potential role in the intrinsically chemotherapy-resistant glioblastomas. In addition, we discuss the mechanisms to sensitize tumors cells to rhTRAIL by combination with the proteasome inhibitor bortezomib.

Introduction

A major challenge in oncology remains the destruction of tumor cells while sparing normal cells. The natural occurring tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL or Apo2L) induces apoptosis in a wide variety of tumor cell lines without causing toxicity to normal cells. Apoptosis is controlled via two major pathways: the intrinsic and extrinsic pathway (Figure 1). TRAIL activates the p53-independent extrinsic pathway by binding to the death receptors (DRs) 4 (TRAIL-R1) and DR5 (TRAIL-R2/KILLER) at the cell surface. This has raised interest in the development of TRAIL receptor targeting drugs for anticancer treatment.

Chemotherapy and radiotherapy initiate apoptosis via the intrinsic pathway. Frequent resistance towards these therapies occurs as a consequence of defects in the intrinsic pathway, for example, mutations in the tumor suppressor gene p53. Combination with agents targeting the TRAIL receptor might circumvent this resistance.

In this review, we describe recent information on the physiological and pathophysiological role of TRAIL and its receptors. We give an update on preclinical and clinical oncological studies with the drugs recombinant human (rh) TRAIL and agonistic antibodies against the DRs. The potential role of these agents in the intrinsically chemotherapy-resistant glioblastomas is highlighted. In addition, we provide a scientific basis for the rationale of combining these compounds with other targeted therapies or cytotoxic therapies in the treatment of cancer, with specific emphasis on the combination of rhTRAIL and the proteasome inhibitor bortezomib.

TRAIL and its receptors

TRAIL is a type II transmembrane protein that forms a homotrimer that binds three receptor molecules on the surface of target cells. A zinc atom in the trimeric ligand is essential for its stability and optimal biological activity (1). The extracellular region of TRAIL can be cleaved from the cell surface by a cysteine protease to form a soluble ligand (2). Both the membrane-bound and soluble form can induce apoptosis in a wide variety of tumor cell lines, but not in most normal cells. TRAIL has five receptors.

Ligation of the membrane-bound receptors DR4 and DR5 initiates the extrinsic apoptotic pathway (Figure 1). TRAIL binding to the two other membrane-bound receptors, decoy receptor 1 (DcR1/TRAIL-R3) and decoy receptor 2 (DcR2/TRAIL-R4), cannot induce apoptosis because they respectively lack an intracellular death domain or have a truncated intracellular death domain. The fifth, soluble receptor osteoprotegerin (OPG) inhibits TRAIL, but has a very low affinity for this ligand at physiological temperatures.

Biological role of endogenous TRAIL and death receptors

TRAIL plays an anti-inflammatory role. Recent data show that endogenous TRAIL induces apoptosis in hepatitis C virus infected hepatocytes and endogenous TRAIL limits the life span of activated leucocytes in a bacterial meningitis model in wildtype mice compared to TRAIL -/- mice (3,4). Moreover, there is growing evidence that TRAIL plays a role in autoimmunity. Blocking TRAIL in mice results in the exacerbation of experimental autoimmune encephalomyelitis, arthritis, and diabetes. In patients with active systemic lupus erythematosus (SLE) T cell membrane expressed TRAIL and soluble TRAIL concentrations as well as TRAIL gene expression in peripheral blood mononuclear cells are markedly elevated. T cell expressed TRAIL in mice results in more CD4+ Th cells that in turn enhances autoreactive B cells and consequently exacerbates SLE (5). Antitumor surveillance is another important property of TRAIL and its receptors (reviewed in (6)). TRAIL-receptor deficiency in mice promotes primary tumor development and results in the faster formation of lymph node metastases without the growth of the primary tumor (7,8*).

Prosurvival role of TRAIL

Recently, several studies indicated that there is also a prosurvival role for the TRAIL-signaling pathway in normal as well as in tumor cells (2). In preclinical models, primary and secondary resistance to rhTRAIL-induced apoptosis does occur. In these cases, stimulation of the DRs results in alternative signaling with ultimate activation and nuclear translocation of NF-κB. This translocation leads to the activation of prosurvival

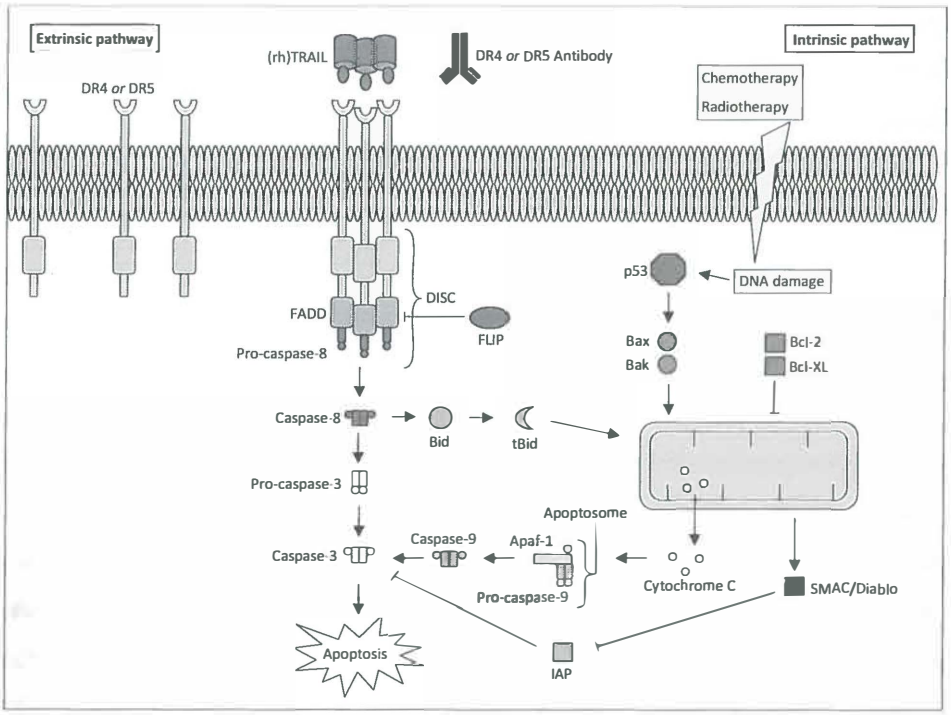


Figure 1. Intrinsic and extrinsic apoptotic pathway.

Upon TRAIL homotrimer binding, the activated death receptors trimerize and recruit Fas-associated death domain (FADD) and the initiator caspase-8. In this death-inducing signaling complex (DISC), caspase-8 is autoactivated by proteolysis and released into the cytosol. This leads to activation of caspase-3 and subsequently apoptosis. FLICE-like inhibitory protein (FLIP) can block this activation. TRAIL can also trigger the intrinsic pathway through caspase-8-mediated cleavage of Bid. Cleaved Bid induces translocation of Bax and/or Bak into the mitochondria leading to cytosolic release of cytochrome *c* and SMAC/Diablo. Cytochrome *c* binds the adaptor proteins Apaf-1 and pro-caspase-9, consequently forming an apoptosome which activates caspase-9. This caspase activates caspase-3 resulting in apoptosis. Bcl-2 and Bcl-XL can inhibit apoptosis by preventing cytochrome c release into the cytosol. The inhibitor of apoptosis proteins (IAPs) blocks caspase activation further downstream. SMAC/Diablo displaces these IAPs, thus promoting apoptosis. The intrinsic pathway can also be triggered after DNA-damage by chemotherapy or radiotherapy.

and antiapoptotic genes. Human pancreatic cancer cells transplanted in severe combined immunodeficiency (SCID) mice showed a striking increase in volume and number of metastases after rhTRAIL treatment (9*). In addition, rhTRAIL induced cell proliferation in cultured small cell lung cancer cells (10). These data reflect a potential back side of TRAIL-receptor targeting agents as anticancer treatment.

Antitumor effect of rhTRAIL and anti-DR4/anti-DR5 antibodies

Administration of rhTRAIL induces increased apoptosis in many cancer cell lines as well as in the inhibition of human tumor xenografts in mice. Moreover, rhTRAIL is nontoxic in nonhuman primates. RhTRAIL combined with chemotherapy, targeted therapies, or radiotherapy results in a synergistic antitumor effect and even restores sensitivity to other treatment modalities in resistant human tumor cells as well as mouse xenograft models. Next to rhTRAIL, several antibodies against the DRs are developed. While rhTRAIL binds all TRAIL receptors, the anti-DR4/anti-DR5 antibodies selectively activate a specific DR. In addition, the serum half-life of rhTRAIL is, at 25 minutes in chimpanzees, much shorter than that of the DR-targeting monoclonal antibodies, which is around two weeks (11). Therefore, the antibodies have more favourable profiles. The preclinical tumoricidal effects seen with these antibodies in tumor cell lines and xenograft models are similar to that observed with rhTRAIL and are also potentiated when combined with other anticancer therapies (6,12).

To select patients who might benefit from selected therapies, it would be very helpful to have predictive biomarkers. The O-glycosylation status of DRs might be of interest. O-linked glycans regulate biochemical and functional properties of cell surface proteins, including apoptosis. O-glycosyltransferase mRNA levels correlate with sensitivity for rhTRAIL in several tumor cell lines. Glycosylation of DRs results in increased TRAIL-induced clustering of DRs and consequently enhances caspase 8 activation and apoptosis (13). However, apart from post-translational modifications in DRs, alterations in proteins more downstream the apoptotic pathway can play a role (14).

The prognostic value of DR expression was addressed in a study concerning 376 stage III colorectal cancer patients. As part of a randomized trial, these patients adjuvantly received chemotherapy. High DR4 expression was associated with a worse prognosis, with a worse disease-free as well as overall survival (15).

Ongoing preclinical exploration: rhTRAIL in malignant gliomas

Malignant gliomas are rapidly progressive brain tumors. Concomitant and adjuvant chemoradiotherapy with temozolomide has become the standard treatment for newly diagnosed glioblastoma resulting in significant, but moderate prolongation of survival. Treatment is still rarely curative and the prognosis of these patients remains dismal with a two-year overall survival of 27% (16). In recent years, research focused on the elucidation of molecular mechanisms underlying glioma development. Mounting evidence, suggesting a role for dysregulation of the apoptotic pathway, has therefore stimulated studies targeting the apoptotic pathway in this setting (17). Preclinical studies with rhTRAIL show induction of apoptosis in glioma cells (18). Efficient transfection of glioblastoma multiforme (GBM) cells with TRAIL-expressing adenovirus results in enhanced cell kill (19). However, resistance toward rhTRAIL-induced cell death is commonly observed in malignant glioma cell lines. To overcome this resistance, rhTRAIL has been combined with various cytotoxic chemotherapeutics and new biological agents. 5-Fluorouracil in combination with rhTRAIL in human glioblastoma xenograft bearing mice enhanced tumor apoptosis and resulted in a prolonged tumor growth delay (20). Moreover, the combination of temozolomide and rhTRAIL results in longer survival in a glioblastoma xenograft model than either agent alone (21). Several new biologicals might be potentially interesting to combine with TRAIL receptor targeting agents. These include heat shock protein inhibitors (HSPis) and histone deacetylase inhibitors (HDACis). HSPs are chaperone proteins that play a role in the regulation of cell growth through different client proteins and thereby have antiapoptotic properties. Downregulation of HSP90 by short-interfering RNA inhibits the recruitment of FLICE-like inhibitory protein (FLIP) and other antiapoptotic proteins to the death-inducing signaling complex (DISC), thereby sensitizing resistant glioma

cells to rhTRAIL-induced apoptosis (22*). HDACi induce apoptosis, tumor cell growth arrest, and differentiation. The HDACi vorinostat inhibits growth of GBM intracranially in mice and is currently evaluated in patients with recurrent GBM in a phase II study (23). Synergistic tumor cell death has been observed in a variety of other human cancer cell lines exposed to both rhTRAIL and HDACi (24). The combination of rhTRAIL with bortezomib is discussed below. It would be of interest to evaluate the combination of these targeted therapies in patients.

Combinatorial TRAIL-receptor directed therapy with bortezomib

Various antitumor agents have been combined with TRAIL to study their sensitizing effects. In particular, combinations with proteasome inhibitors have recently gained substantial interest. The first proteasome inhibitor to be granted approval was bortezomib, a selective and reversible inhibitor of the proteasomal degradation of proteins.

The combination of rhTRAIL and bortezomib, has been studied in different cancer cell lines, such as non-small cell lung cancer (NSCLC), malignant glioma, hepatocellular carcinoma, colon, and pancreatic cancer (25-28). In many rhTRAIL-resistant cancer cell lines, bortezomib is able to sensitize these cells to rhTRAIL-induced apoptosis.

Combined exposure to rhTRAIL and bortezomib results in the activation of caspase-8 and caspase-3. Also caspase-9 can be activated indicating cross-talk between the extrinsic and intrinsic pathway (Figure 1) (26-28). Bortezomib enhances cell surface expression of DR4 and especially DR5 (25-29). However, this mechanism can only partly explain the observed rhTRAIL sensitization (25-27). Sensitized tumor cells demonstrated stronger DISC formation upon rhTRAIL incubation, with increased Fas-associated death domain (FADD) and caspase-8 recruitment (26). The effect of bortezomib on FLIP levels, however, remains controversial. Some studies report no difference in FLIP levels (28), while others find an up-regulation (27), or down-regulation (25,26).

As stated before, rhTRAIL can activate the NF-κB survival pathway, which then contributes to rhTRAIL resistance. Bortezomib co-incubation is able to effectively inhibit TRAIL-induced NF-κB activation, thereby blocking this survival route (28).

No toxicity for either single agent rhTRAIL or bortezomib was found in primary human hepatocytes (PHHs), although combined administration results in some TRAIL-induced apoptosis in these cells. However, bortezomib and rhTRAIL induced apoptosis in hepatoma, colon and pancreatic cancer cell lines occurs at a more than 40-fold lower bortezomib concentration (25 nM) than in PHHs. This suggests a therapeutic window for combination therapy with bortezomib and rhTRAIL (26).

Next to rhTRAIL, agonistic DR antibodies are also studied in combination with bortezomib. These combinations enhance both the extrinsic and intrinsic pathways in non-Hodgkin's lymphoma (NHL) cell lines (29). In mice bearing human lymphoma cell line xenografts, the combination of bortezomib and either DR-antibody shows a greater clearance of lymphoma cells from ascites and a trend to prolonged time until recurrence of lymphoma cells, indicating enhanced tumor cell death with the combination therapy (29). Currently a phase 2 study is ongoing, combining the DR4 antibody mapatumumab with bortezomib in multiple myeloma patients.

Clinical application of rhTRAIL and anti-DR4/anti-DR5 monoclonal antibodies

RhTRAIL and several anti-DR4/anti-DR5 monoclonal antibodies have been evaluated in several clinical trials (Table 1). In phase 1 and 2 studies patients received rhTRAIL doses up to 15 mg/kg intravenous (i.v.) for five consecutive days. Preliminary results showed that rhTRAIL appeared to be well tolerated. The serum half-life is approximately 36 min at 8 mg/kg. One partial response was seen in a patient with a chondrosarcoma. (R Herbst, abstract in *J Clin Oncol* 2006, 24:3013). In a phase 1b study evaluating the safety of rituximab in combination with rhTRAIL in patients with NHL, three of five evaluable patients showed responses without apparent toxicity (L Yee, abstract in *J Clin Oncol* 2007, 25:8078). This combination is now further explored in a phase 2 study. At present, six agonistic monoclonal antibodies against the DRs are evaluated in clinical trials. Mapatumumab is the only one that specifically binds to DR4. In a phase 1 study

with this agent 49 patients received doses up to 10 mg/kg i.v. every two weeks. No significant toxicity was seen and the maximum tolerated dose has not been reached. However, the highest dose studied at present is far above the predicted effective dose of 6 mg/kg, on the basis of preclinical models. The mean terminal elimination half-life is 18.8 days, which make dosing every three weeks feasible (30*).

Subsequently, three phase 2 studies were initiated in patients with NHL, colorectal cancer, and advanced NSCLC. Among the 40 NHL patients, 1 complete and 2 partial responses were observed (A Younes *et al*., abstract 409, 47th ASH Annual Meeting, Atlanta, December 2005). In the NSCLC study 32, most heavily pretreated patients were enrolled at 10 mg/kg every 3 weeks. No responses were seen, however, up to 29% had stable disease for on average 2.3 months (31). Mapatumumab in combination with paclitaxel and carboplatin was considered safe in a phase 1b study. 4/28 patients experienced a confirmed partial response, including 3 patients with NSCLC (L Chow, abstract in *J Clin Oncol* 2006, 24:2515). A phase 2 study with this combination in NSCLC patients has been initiated. In a phase 1b study, mapatumumab could be administered safely up to 30 mg/kg every three weeks in combination with gemcitabine and cisplatin. No alterations in the pharmacokinetic profiles were observed. 11/45 patients experienced a partial response and 13/45 patients achieved stable disease more than 18 weeks. (Oldenhuis *et al*., abstract 3540, 44th ASCO Annual Meeting, Chicago, June 2008)

One of the DR5-targeting antibodies, lexatumumab, was found to be safe and well tolerated at the maximum tolerated dose of 10 mg/kg. The average serum half-life in this study was 16.4 days. The best observed response was stable disease in 12 out of 37 patients treated with a median duration of 4.5 months (32). Lexatumumab has also been evaluated in combination with several chemotherapy regimens. Reports of the other anti-DR agents summarized in table 1 are awaited.

Table 1. Overview of TRAIL receptor targeting agents in clinical evaluation.

Agent (target)	Clinical development	Combination	Tumor type
rhTRAIL (DR4 and DR5)	Phase 1b	Monotherapy	CRC
	Phase 2	±Rituximab	NHL
		Carboplatin/paclitaxel ± bevacizumab	NSCLC
Mapatumumab (DR4)	Phase 1b	Cisplatin/gemcitabine	Various
	Phase 2	Monotherapy	CRC, NHL, NSCLC
		Paclitaxel/carboplatin	NSCLC
		Bortezomib	Multiple myeloma
Lexatumumab (DR5)	Phase 1	Monotherapy	Various
	Phase 1b	Gemcitabine	Various
		Pemetrexed	Various
		Doxorubicin	Various
		FOLFIRI	Various
Apomab (DR5)	Phase 2	Monotherapy	Chondrosarcoma
		Rituximab	NHL
		Carboplatin/paclitaxel/ bevacizumab	NSCLC
		Cetuximab/irinotecan	CRC
AMG655 (DR5)	Phase 1b/2	Gemcitabine	Pancreatic cancer
		FOLFOX6/bevacizumab	CRC
		Panitumumab	CRC
		Doxorubicin	Soft tissue sarcoma
		Paclitaxel/carboplatin	NSCLC
CS 1008 (DR5)	Phase 2	Gemcitabine	Pancreatic cancer
LBY135 (DR5)	Phase 1	±Capecitabine	Various

CRC: colorectal cancer; NHL: non-Hodgkin's lymphoma; NSCLC: non-small cell lung cancer; FOLFIRI: leucovorin, fluorouracil and irinotecan; FOLFOX: leucovorin,fluorouracil and oxaliplatin.
(www.hgsi.com/hgs-etr1.html; www.clinicaltrials.gov; www.amgen.com/science/pipe.jsp; www.novartisoncology.com/research-innovation/pipeline.jsp)

Conclusions

Interests in TRAIL, boosted by its unique property to selectively induce apoptosis in tumor cells, have resulted in the first clinical application of TRAIL-receptor targeting agents in the past years. Concerns about liver toxicity and autoimmune phenomena have been so far abrogated by clinical trials. Both rhTRAIL and TRAIL-receptor antibodies appear to be safe. Single agent response rates, however, are low. TRAIL-receptor targeting agents do not appear to be potent enough and might need sensitizing agents to induce tumor cell apoptosis. TRAIL-receptor targeting agents are now being studied in combination with sensitizing cytotoxic therapies and other targeted therapies. Although combination of radiotherapy with DR-targeting antibodies have shown synergistic antitumor effects in xenograft mice, no clinical studies with this combination have been initiated till date (12).

Little knowledge is available concerning the biodistribution of the TRAIL-receptor-targeting agents in man and it is therefore not clear if these agents penetrate the tumor. In an attempt to visualize the tumor, a SPECT imaging study with the radioactively labeled DR4 antibody mapatumumab has commenced.

Overall, however, future research will focus on combination strategies. Furthermore, incorporation of potential predictive biomarker analyses into clinical trials is needed. Together, this could guide patient-tailored therapy.

Conflict of interest statement

CNAM Oldenhuis and EGE de Vries performed a study with mapatumumab (Human Genome Sciences) and performed a study with LBY135 (Novartis). The University Medical Center Groningen receives the study drugs and financial support to perform the studies.

Acknowledgment

This research is supported by Grant 2006-3567 of the Dutch Cancer Society.

References and recommended reading

Papers of particular interest have been highlighted as:
* of special interest

1. Hymowitz SG, O'Connell MP, Ultsch MH, Hurst A, Totpal K, Ashkenazi A, de Vos AM, Kelley RF: A unique zinc-binding site revealed by a high-resolution X-ray structure of homotrimeric Apo2L/TRAIL. Biochemistry 2000, 39:633-640.
2. Malhi H, Gores GJ: TRAIL resistance results in cancer progression: a TRAIL to perdition? Oncogene 2006, 25:7333-7335.
3. Hoffmann O, Priller J, Prozorovski T, Schulze-Topphoff U, Baeva N, Lunemann JD, Aktas O, Mahrhofer C, Stricker S, Zipp F, Weber JR: TRAIL limits excessive host immune responses in bacterial meningitis. J.Clin.Invest 2007, 117:2004-2013.
4. Zhu H, Dong H, Eksioglu E, Hemming A, Cao M, Crawford JM, Nelson DR, Liu C: Hepatitis C virus triggers apoptosis of a newly developed hepatoma cell line through antiviral defense system. Gastroenterology 2007, 133:1649-1659.
5. Rus V, Nguyen V, Puliaev R, Puliaeva I, Zernetkina V, Luzina I, Papadimitriou JC, Via CS: T cell TRAIL promotes murine lupus by sustaining effector CD4 Th cell numbers and by inhibiting CD8 CTL activity. J.Immunol. 2007, 178:3962-3972.
6. Duiker EW, Mom CH, de Jong S, Willemse PH, Gietema JA, van der Zee AG, de Vries EG: The clinical trail of TRAIL. Eur.J.Cancer 2006, 42:2233-2240.
7. Finnberg N, Klein-Szanto AJ, El-Deiry WS: TRAIL-R deficiency in mice promotes susceptibility to chronic inflammation and tumorigenesis. J.Clin.Invest 2008, 118:111-123.
*8. Grosse-Wilde A, Voloshanenko O, Bailey SL, Longton GM, Schaefer U, Csernok AI, Schutz G, Greiner EF, Kemp CJ, Walczak H: TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. J.Clin.Invest 2008, 118:100-110.
The authors generated TRAIL-receptor deficient mice and subsequently initiated skin tumors. The number, rate of appearance and malignant transformation rate of benign papilloma did not differ between TRAIL-R-/- mice compared to TRAIL-R+/- and TRAIL-R+/+ mice. However TRAIL-R -/- mice developed earlier and significant more lymph node metastases, suggesting a protective role for the TRAIL-R in mice.
*9. Trauzold A, Siegmund D, Schniewind B, Sipos B, Egberts J, Zorenkov D, Emme D, Roder C, Kalthoff H, Wajant H: TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. Oncogene 2006, 25:7434-7439.
In this paper, the authors inoculated SCID mice with human pancreatic cancer cells overexpressing Bcl-XL, thereby inhibiting TRAIL-induced apoptosis. Upon rhTRAIL treatment a 5.8-fold increase in the tumor volume and a significant increase in the number of liver metastases were found compared to saline-treated mice.
10. Belyanskaya LL, Ziogas A, Hopkins-Donaldson S, Kurtz S, Simon HU, Stahel R, Zangemeister-Wittke U: TRAIL-induced survival and proliferation of SCLC cells is mediated by ERK and dependent on TRAIL-R2/DR5 expression in the absence of caspase-8. Lung Cancer 2008, 60:355-365
11. Kelley SK, Harris LA, Xie D, Deforge L, Totpal K, Bussiere J, Fox JA: Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of in vivo efficacy, pharmacokinetics, and safety. J.Pharmacol.Exp.Ther. 2001, 299:31-38.
12. Marini P, Denzinger S, Schiller D, Kauder S, Welz S, Humphreys R, Daniel PT, Jendrossek V, Budach W, Belka C: Combined treatment of colorectal tumours with agonistic TRAIL receptor antibodies HGS-ETR1 and HGS-ETR2 and radiotherapy: enhanced effects in vitro and dose-dependent growth delay in vivo. Oncogene 2006, 25:5145-5154.
13. Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, Lee D, von Goetz M, Yee SF, Totpal K, et al.: Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nat.Med. 2007, 13:1070-1077.
14. Carlo-Stella C, Lavazza C, Locatelli A, Vigano L, Gianni AM, Gianni L: Targeting TRAIL agonistic receptors for cancer therapy. Clin.Cancer Res. 2007, 13:2313-2317.

15. Van Geelen CM, Westra JL, de Vries EG, Boersma-van EW, Zwart N, Hollema H, Boezen HM, Mulder NH, Plukker JT, de Jong S, et al.: Prognostic significance of tumor necrosis factor-related apoptosis-inducing ligand and its receptors in adjuvantly treated stage III colon cancer patients. J.Clin.Oncol. 2006, 24:4998-5004.
16. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, et al.: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N.Engl.J.Med. 2005, 352:987-996.
17. Lefranc F, Brotchi J, Kiss R: Possible future issues in the treatment of glioblastomas: special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis. J.Clin.Oncol. 2005, 23:2411-2422.
18. Hao C, Beguinot F, Condorelli G, Trencia A, Van Meir EG, Yong VW, Parney IF, Roa WH, Petruk KC: Induction and intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apotosis in human malignant glioma cells. Cancer Res. 2001, 61:1162-1170.
19. Wohlfahrt ME, Beard BC, Lieber A, Kiem HP: A capsid-modified, conditionally replicating oncolytic adenovirus vector expressing TRAIL leads to enhanced cancer cell killing in human glioblastoma models. Cancer Res. 2007, 67:8783-8790.
20. Lee KC, Hamstra DA, Bhojani MS, Khan AP, Ross BD, Rehemtulla A: Noninvasive molecular imaging sheds light on the synergy between 5-fluorouracil and TRAIL/Apo2L for cancer therapy. Clin.Cancer Res. 2007, 13:1839-1846.
21. Saito R, Bringas JR, Panner A, Tamas M, Pieper RO, Berger MS, Bankiewicz KS: Convection-enhanced delivery of tumor necrosis factor-related apoptosis-inducing ligand with systemic administration of temozolomide prolongs survival in an intracranial glioblastoma xenograft model. Cancer Res. 2004, 64:6858-6862.
*22. Panner A, Murray JC, Berger MS, Pieper RO: Heat shock protein 90alpha recruits FLIPS to the death inducing signaling complex and contributes to TRAIL resistance in human glioma. Cancer Res. 2007, 67:9482-9489.
The authors show that high levels of the antiapoptotic protein FLIP alone are not sufficient enough to inhibit apoptosis. HSP90α is needed to serve as chaperone protein by recruiting FLIP to the DISC and inhibiting apoptosis in human glioma cells.
23. Yin D, Ong JM, Hu J, Desmond JC, Kawamata N, Konda BM, Black KL, Koeffler HP: Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor: effects on gene expression and growth of glioma cells in vitro and in vivo. Clin.Cancer Res. 2007, 13:1045-1052.
24. Fulda S: Modulation of TRAIL-induced apoptosis by HDAC inhibitors. Curr.Cancer Drug Targets. 2008, 8:132-140.
25. Koschny R, Holland H, Sykora J, Haas TL, Sprick MR, Ganten TM, Krupp W, Bauer M, Ahnert P, Meixensberger J, Walczak H: Bortezomib sensitizes primary human astrocytoma cells of WHO grades I to IV for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. Clin.Cancer Res. 2007, 13:3403-3412.
26. Koschny R, Ganten TM, Sykora J, Haas TL, Sprick MR, Kolb A, Stremmel W, Walczak H: TRAIL/bortezomib cotreatment is potentially hepatotoxic but induces cancer-specific apoptosis within a therapeutic window. Hepatology. 2007, 45:649-658.
27. Liu X, Yue P, Chen S, Hu L, Lonial S, Khuri FR, Sun SY: The proteasome inhibitor PS-341 (bortezomib) upregulates DR5 expression leading to induction of apoptosis and enhancement of TRAIL-induced apoptosis despite up-regulation of c-FLIP and survivin expression in human NSCLC cells. Cancer Res. 2007, 67:4981-4988. bb
28. Voortman J, Resende TP, Abou El Hassan MA, Giaccone G, Kruyt FA: TRAIL therapy in non-small cell lung cancer cells: sensitization to death receptor-mediated apoptosis by proteasome inhibitor bortezomib. Mol.Cancer Ther. 2007, 6:2103-2112.
29. Smith MR, Jin F, Joshi I: Bortezomib sensitizes non-Hodgkin's lymphoma cells to apoptosis induced by antibodies to tumor necrosis factor related apoptosis-inducing ligand (TRAIL) receptors TRAIL-R1 and TRAIL-R2. Clin.Cancer Res. 2007, 13:5528s-5534s.
*30. Tolcher AW, Mita M, Meropol NJ, von Mehren M, Patnaik A, Padavic K, Hill M, Mays T, McCoy T, Fox NL, et al.: Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. J.Clin.Oncol. 2007, 25:1390-1395.

A phase 1 dose escalation study was performed with a fully human monoclonal antibody targeting DR4. Initially, patients received a single dose. After the confirmation of safety, dose and frequency of administration were carefully increased to 10 mg/kg every 14 days. Most common and mild adverse events were fatigue, fever, and myalgia. The authors showed that this antibody could be administered safely up to 10 mg/kg i.v. every two weeks.

31. Greco FA, Bonomi P, Crawford J, Kelly K, Oh Y, Halpern W, Lo L, Gallant G, Klein J: Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer. Lung Cancer 2008, 61:82-90.
32. Plummer R, Attard G, Pacey S, Li L, Razak A, Perrett R, Barrett M, Judson I, Kaye S, Fox NL, et al.: Phase 1 and pharmacokinetic study of lexatumumab in patients with advanced cancers. Clin.Cancer Res. 2007, 13:6187-6194.

CHAPTER 3

Mapatumumab, a fully human agonistic monoclonal antibody that targets TRAIL-R1, in combination with gemcitabine and cisplatin: a phase I study

CH Mom[1], J Verweij[2], CNAM Oldenhuis[1], JA Gietema[1], NL Fox[3], R Miceli[3], FALM Eskens[2], WJ Loos[2], EGE de Vries[1], S Sleijfer[2].

[1]Department of Medical Oncology, University Medical Center Groningen, The Netherlands; [2]Department of Medical Oncology, Erasmus University Medical Center Rotterdam, The Netherlands; [3]Human Genome Sciences, Rockville, MD, USA.

Clinical Cancer Research. 2009;15:5584-90.

Note: Presented in part at the AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics, Philadelphia, PA, Nov 14-18, 2005; EORTC-NCI-AACR International Conference on Molecular Targets and Cancer Therapeutics, Prague, Czech Republic, Nov 7-10, 2006; and the 44th Annual Meeting of the American Society of Clinical Oncology, Chicago, IL, May 30- June 3, 2008.

Abstract

Purpose: To evaluate the safety, tolerability, pharmacokinetics and antitumor activity of mapatumumab, a fully human monoclonal antibody targeting tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1), in combination with gemcitabine and cisplatin.

Experimental design: Patients with advanced solid tumors received gemcitabine 1250 mg/m^2 i.v. on days 1 and 8, and cisplatin 80 mg/m^2 i.v. on day 1 of each 21-day cycle. Escalating mapatumumab doses were administered i.v. every 21 days. Toxicity was evaluated and pharmacokinetic analysis of plasma mapatumumab, gemcitabine, 2-difluoro-2-deoxyuridine, unbound and total platinum was performed. TRAIL-R1 tumor expression was determined immunohistochemically.

Results: 49 patients received mapatumumab (1 mg/kg (n=4), 3 mg/kg (n=7), 10 mg/kg (n=12), 20 mg/kg (n=13) or 30 mg/kg (n=13)). A median of 6 cycles (range 1-48) was administered. The adverse events most commonly observed reflect the toxicity profile of gemcitabine and cisplatin. Dose-limiting toxicities (DLT) were seen in 3/12 patients at 10 mg/kg, consisting of grade 3 transaminitis, neutropenic fever and grade 4 thrombocytopenia. At 20 mg/kg 2/12 patients had DLTs, including grade 4 thrombocytopenia and grade 4 fatigue. The maximum tolerated dose was not reached. Pharmacokinetic interactions have not been observed. 12 patients had a partial response, and 25 patients showed stable disease with a median duration of 6 months.

Conclusions: Mapatumumab in combination with gemcitabine and cisplatin is safe and well tolerated at doses up to 30 mg/kg. Further studies of this combination are warranted.

Introduction

The Tumor Necrosis Factor (TNF)-related Apoptosis-Inducing Ligand (TRAIL) pathway is an attractive target for antitumor therapy, since activation of death receptors on the cell surface results in stimulation of the intracellular signaling routes leading to apoptosis. The naturally occurring ligand TRAIL is a member of the TNF superfamily which can bind to 5 different receptors, including TRAIL receptors 1 (TRAIL-R1, DR4) and 2 (TRAIL-R2, DR5), through which TRAIL transmits its apoptotic signal (1,2). For yet unknown reasons TRAIL, in preclinical models, selectively induces apoptosis in tumor cells without toxic effects on normal cells.

Mapatumumab (TRM-1, ETR1) is a fully human immunoglobulin G1 lambda (IgG1λ) agonistic monoclonal antibody to TRAIL-R1 competing with TRAIL for binding to TRAIL-R1. Single agent mapatumumab induces apoptosis in a range of cancer cell lines and inhibits tumor growth in various human tumor mouse xenograft models (3). Expression of TRAIL-R1 is frequently observed in human tumors, including pancreatic, ovarian, colorectal, gastric, uterine, lung and breast carcinomas (4).

In two phase I studies in patients with solid tumors, single agent mapatumumab was well tolerated and adverse events were generally mild to moderate in severity (5;6). The maximum tolerated dose (MTD) in these studies was not reached with 10 mg/kg every 14 days (6) and 20 mg/kg every 28 days (5). Pharmacokinetic (PK) results showed a mean terminal elimination half-life of 18-21 days. Additionally, in phase II studies with mapatumumab in patients with non-small cell lung cancer (NSCLC), colorectal cancer (CRC) and non-Hodgkin's lymphoma (NHL), a similar toxicity profile was observed (7-9). In the NSCLC and CRC studies, stable disease was the best observed response in 30% of the patients. Three patients with follicular lymphoma experienced a tumor response.

Combination therapy may synergistically enhance antitumor activity because mapatumumab induces apoptosis via the extrinsic pathway, while chemotherapy results in cell death by activating the intrinsic pathway. Accordingly, the addition of mapatumumab to gemcitabine or cisplatin resulted in increased cytotoxicity in various human tumor cell lines. Furthermore, in a human NSCLC (H460) xenograft model,

combining cisplatin and mapatumumab showed increased tumor growth inhibition compared to either agent alone (10). We therefore performed a phase I study to evaluate the safety and tolerability of escalating doses of mapatumumab in combination with gemcitabine and cisplatin in patients with solid tumors. Secondary objectives were to determine plasma mapatumumab concentrations, to assess the influence of mapatumumab on plasma gemcitabine and cisplatin PK, to evaluate disease response and to assess TRAIL-R1 expression in tumors of participating patients.

Patients and methods

Eligibility criteria

Eligible for the study were patients with a histologically or cytologically confirmed advanced solid malignancy for whom no standard therapy options were available or for whom the combination of gemcitabine and cisplatin was considered an appropriate treatment. Additional eligibility criteria included: age ≥ 18 years; Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1; estimated life expectancy ≥ 3 months; adequate bone marrow and renal function, AST and ALT ≤ 2.5 fold upper limit of normal (ULN); no previous chemotherapy, immunotherapy, radiotherapy or hormonal therapy within 3 weeks; no treatment with monoclonal antibodies within previous 3 weeks (murine or chimeric) or 8 weeks (human or humanized); no investigational agents within 4 weeks. Specific exclusion criteria included: known positive human immunodeficiency virus status; known chronic or acute viral hepatitis; clinical signs of brain metastases; hearing loss requiring the use of a hearing aid; neuropathy grade ≥ 2; myocardial infarction, cerebrovascular accident or ≥ NYHA Class III congestive heart failure within 6 months prior to study entry.

The study was approved by the local Ethics Committees and the competent regulatory authorities. All patients provided written informed consent.

Study design

A cycle was defined as 21 days. Mapatumumab was administered at escalating dose levels (1, 3, 10, 20 and 30 mg/kg). At each dose level 3-4 patients were enrolled initially. Patients were considered to be evaluable for toxicity if they had completed one full cycle. Dose escalation decisions were made based on the safety assessments of all patients in the dose cohort using the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI-CTCAE) version 3.0. Dose escalation was considered if < 33% of the patients experienced dose-limiting toxicity (DLT). If one of 3-4 patients experienced a DLT, the dose level was expanded to a total of 6 evaluable patients. If a DLT was observed in ≥ 2 out of 6 patients in a cohort the maximum tolerated dose (MTD) was exceeded. To further characterize safety at a certain dose level, up to 12 additional patients could be included.

By the initial DLT criteria, electrolyte disturbances secondary to inadequate antiemetic therapy and chemotherapy-induced transient liver enzyme elevations were considered dose-limiting. The protocol was amended after inclusion of the 5th patient in the 10 mg/kg cohort in order to consider only those events as DLTs that were at least possibly related to mapatumumab or to its interaction with gemcitabine and/or cisplatin. DLTs were thereafter defined as: grade 4 neutropenia lasting > 7 days, febrile neutropenia, grade 4 thrombocytopenia; and grade 3 or 4 non-hematologic adverse events, with the following qualifications: grade ≥ 3 transaminase elevations that did not resolve to ≤ grade 1 before cycle 2, grade ≥ 3 nausea and vomiting despite optimal antiemetic treatment, persistent grade ≥ 2 neuropathy, serum creatinine ≥ 2 times ULN, and grade 4 fatigue. Electrolyte disturbances due to inadequately treated vomiting were not considered dose-limiting if this had resolved prior to cycle 2. All DLTs described in this article are DLTs according to the amended protocol.

Patients could receive a maximum of 6 chemotherapy cycles. Thereafter, in the absence of disease progression, mapatumumab monotherapy could be continued. Patients were withdrawn from the study in case of disease progression, unacceptable toxicity or refusal of treatment.

Drug administration

Mapatumumab (Human Genome Sciences Inc, Rockville, MD) was administered i.v. in 250 mL 0.9% saline over 2 hours on day 2 of cycle 1 and on day 1 of cycles 2-6 after gemcitabine and cisplatin administration.

Gemcitabine (Eli Lilly, Indianapolis, IN) 1250 mg/m^2 was administered i.v. over 30 minutes on days 1 and 8 of each cycle. Cisplatin (Pharmachemie, Haarlem, The Netherlands) 80 mg/m^2 in 1000 mL of 0.9% saline was infused i.v. over 3 hours following gemcitabine on day 1 of each cycle.

No dose modifications were allowed for mapatumumab. Chemotherapy doses were reduced for severe side effects considered to be at least possibly related to chemotherapy or for the patient's safety in the opinion of the investigator. At the beginning of each cycle the absolute neutrophil count (ANC) had to be ≥ 1,500/μL and platelets ≥ 100,000/μL. Treatment could be delayed up to 2 weeks for hematologic recovery. Chemotherapy doses were reduced when treatment was delayed for > 1 week. The day 8 gemcitabine was omitted in case of ANC < 1,000/μL and platelets < 75,000/μL.

Pretreatment and follow-up studies

Prior to therapy a complete medical history, physical examination, electrocardiography, chest X-ray, laboratory evaluations (including blood chemistry, hematology, and urinalysis), and CT scan or MRI for disease assessment were performed. Vital signs were taken prior to administration of the study agents, every 30 minutes during mapatumumab administration and 1, 2, 3 and 4 hours following completion of mapatumumab. Patients were evaluated for toxicity and laboratory values before the start of every cycle, on days 5 ± 1, 8, 11 ± 1 and 15 of each cycle and on days 2 and 3 of cycle 1. Response was assessed after every 2 cycles by Response Evaluation Criteria in Solid Tumors (11). If patients continued on mapatumumab monotherapy, tumor evaluations were performed every 3 cycles.

Pharmacokinetics and immunogenicity

Serial blood samples for plasma gemcitabine, cisplatin and mapatumumab concentration measurements were collected prior to dosing and over a 26 hour period following the start of the infusion of the respective agents in cycles 1 and 2. In addition, mapatumumab samples were collected on day 15 of cycle 2, days 1 and 15 of cycle 4 and 6, day 15 and 29 of the final cycle, and during mapatumumab monotherapy prior to dosing every third cycle. Serum samples for immunogenicity were also collected prior to each cycle to determine plasma mapatumumab concentrations and anti-mapatumumab antibodies (6).

Plasma concentrations of gemcitabine and its inactive metabolite 2-difluoro-2-deoxyuridine (dFdU) were measured using a validated HPLC with diode array detection, and analysis of unbound platinum and total platinum were performed using an atomic absorption spectrophotometer (12). To determine PK parameters, plasma concentrations of gemcitabine, dFdU, cisplatin-derived unbound and total platinum were subjected to non-compartmental analysis with WinNonlin Enterprise, version 5.0.1 (Pharsight Corporation, Cary, NC) using nominal dose and actual times postdose according to standard methods (13). Differences in PK parameters between cycle 1 (prior to mapatumumab treatment) and cycle 2 (with mapatumumab treatment) were assessed by a 95% CI for each cohort. Plasma mapatumumab concentration results were compared to a predicted range of concentrations based on phase 1 study results in subjects with solid tumors who received mapatumumab as a monotherapy (5, 6).

Immunohistochemical staining of tumor tissue

3 μm thick archival tumor tissue sections from patients were cut from paraffin blocks and deparaffinized in xylene. Slides were immunohistochemically stained for TRAIL-R1 (14). Thereafter, slides were reviewed by light microscopy and scored by two investigators (CO (deblinded for clinical outcome), JB (blinded for clinical outcome)). Intensity of TRAIL-R1 staining was scored in four categories (no, low, moderate, and strong).

Table 1. Patient characteristics.

Characteristic	No. of patients (n=49)
Age	
Median	53
Range	34-72
Sex	
Male	32
Female	17
ECOG performance status	
0	18
1	31
Prior treatment	
Systemic*	14
Surgery	29
Radiotherapy	8
Tumor types	
Pancreatic cancer	19
(A)CUP	9
Esophageal cancer	4
NSCLC	3
Cholangiocarcinoma	3
Melanoma	2
SCLC	2
Bladder cancer	1
Carcinoid	1
Gallbladder	1
Gastric	1
Head-neck	1
Renal	1
Thymus	1

Abbreviations: (A)CUP, (adeno)carcinoma of unknown primary; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

* Includes chemotherapy, immunotherapy, and hormonal therapy.

Results

General

Patient characteristics are shown in Table 1. From November 2004 to 31 January 2009, 49 patients received a total of 209 cycles of combination treatment (median 5, range 1-6). Additionally, 15 patients received 124 cycles of mapatumumab monotherapy (median 6, range 2-42). The number of patients treated at each dose level is shown in Table 2. At 20 mg/kg one patient experienced a cerebrovascular accident on day 5 of cycle 1, most likely related to cisplatin. In the 30 mg/kg cohort one patient experienced an epileptic seizure due to prior unknown brain metastases and went off study on day 8 of the first cycle. Both patients were replaced because the toxicity data for cycle 1 were incomplete.

Table 2. Mapatumumab dose levels and DLTs as a function of dose.

Dose level (mg/kg)	No. of patients	Median no. of cycles (range)	No. of patients with DLT
1	4	6 (3-12)	0
3	7	6 (3-18)	0
10	12	6 (1-21)	3
20	13	4 (1-48)*	2
30	13	5 (1-15)	0

Abbreviation: DLT, dose-limiting toxicity.

*One patient is ongoing and received 48 cycles as of 31 January 2009.

Toxicity

The most frequent chemotherapy-related and/or mapatumumab-related adverse events are listed in Table 3. Nausea and vomiting was frequently observed. These are well known side effects of cisplatin and gemcitabine and were therefore considered most likely caused by the chemotherapy. Hematologic toxicity, including thrombocytopenia, neutropenia and anemia, were considered to be most likely related to cisplatin and gemcitabine, although anemia may also have been cancer-related. Hematologic toxicity caused treatment delays, dose reductions and/or omissions of

day 8 gemcitabine at all mapatumumab dose levels. The dose intensity of cisplatin and gemcitabine was maintained at the highest dose level of mapatumumab (Table 4). Grade 3 or 4 elevations of transaminases was observed in 8 patients (16%). Three of these were reported as adverse events at least probably related to gemcitabine; 1 of the 3 was also considered possibly related to cisplatin and mapatumumab.

Table 3. Number of patients with study drugs-related* adverse events by MedDRA preferred term and maximum toxicity grade occurring in ≥ 10% of all patients (n=49).

Adverse event	Mild		Moderate		Severe		Life-threatening		All active	
	No.	%	No.	%	No.	%	No.	%	No.	%
Nausea	20	40.8	22	44.9	4	8.2	0		46	93.9
Vomiting	18	36.7	18	36.7	3	6.1	0		39	79.6
Fatigue	10	20.4	22	44.9	4	8.2	1	2.0	37	75.5
Thrombocytopenia	2	4.1	5	10.2	12	24.5	7	14.3	26	53.1
Neutropenia	0		4	8.2	12	24.5	9	18.4	25	51.0
Tinnitus	10	20.4	12	24.5	1	2.0	0		23	46.9
Anemia	0		17	34.7	4	8.2	0		21	42.9
Leukopenia	0		6	12.2	11	22.4	3	6.1	20	40.8
Alopecia	10	20.4	8	16.3	0		0		18	36.7
Peripheral sensory neuropathy	12	24.5	3	6.1	0		0		15	30.6
Diarrhea	7	14.3	4	8.2	1	2.0	0		12	24.5
Dysgeusia	8	16.3	2	4.1	0		0		10	20.4
Anorexia	9	18.4	0		0		0		9	18.4
Stomatitis	9	18.4	0		0		0		9	18.4
Hypomagnesemia	4	8.2	3	6.1	1	2.0	0		8	16.3
Hypokalemia	3	6.1	0		2	4.1	0		5	10.2
Paresthesia	4	8.2	1	2.0	0		0		5	10.2

Abbreviation: MedDRA, medical dictionary for regulatory activities.

*possibly, probably or definitely related to gemcitabine, cisplatin and/or mapatumumab.

Table 4. Dose intensity of gemcitabine and cisplatin.

Map dose level in mg/kg	No. of pts	Median no. of cycles (range)		No. of pts with delay (both gem and cis)	No. of administrations with delay (due to hematologic recovery)		Relative dose intensity (%)*	
		gem	cis		gem	cis	gem	cis
1	4	6 (4-6)	6 (3-6)	1	3 (1)	3 (1)	87.2	94.8
3	7	6 (3-6)	6 (3-6)	4	7 (3)	6 (3)	77.1	86.9
10	12	6 (1-6)	4.5 (1-6)	9	17 (15)	16 (14)	71.4	82.7
20	13	4 (1-6)	4 (1-6)	6	12 (10)	12 (10)	68.4	92.3
30	13	5 (1-6)	4 (1-6)	4	8 (7)	8 (7)	82.1	92.5
All	49	5 (1-6)	4 (1-6)	24	47 (36)	45 (35)	75.5	89.4

Abbreviations: map, mapatumumab; pts, patients; gem, gemcitabine; cis, cisplatin.

*Relative dose intensity = dose intensity in mg/m^2 / planned dose intensity in mg/m^2 x 100%

Dose-limiting toxicity (Table 2)

In the 1 mg/kg and 3 mg/kg cohort no DLTs occured. At 10 mg/kg one patient experienced a grade 3 ALT elevation. However, this patient had an elevated ALT > 2.5 times ULN at baseline (88 U/L; ULN 30 U/L) and therefore did not meet the inclusion criteria. The grade 3 ALT elevation was regarded as probably related to gemcitabine and possibly related to mapatumumab. The 10 mg/kg cohort after expansion to 6 patients showed no additional DLTs. During expansion of the 10 mg/kg cohort two additional patients experienced a DLT. One patient developed neutropenic fever; the other, grade 3 ALT and AST elevations, and grade 4 thrombocytopenia. Thus, 3 out of 12 patients experienced a DLT at 10 mg/kg.

In the 20 mg/kg cohort DLTs were observed in 2 out of 12 evaluable patients. One patient had a grade 4 fatigue and one patient grade 4 thrombocytopenia.

No DLTs occurred in the 30 mg/kg cohort.

Pharmacokinetics

Individual subjects' plasma mapatumumab concentrations for each cohort of this study, along with predicted concentrations based on phase I PK study results, are

illustrated in Figure 1. There was substantial overlap between the observed concentrations and the predicted concentration ranges, at all dose levels.

The mean (± SD) plasma gemcitabine, dFdU, unbound platinum and total platinum concentration-time profiles, pooled from all cohorts, are shown in Figure 2. For each compound, concentrations for the 1st treatment cycle are virtually superimposable or within 1 SD of the respective mean for the 2nd treatment cycle.

Mean (with 95% CI) exposure in cycle 1 and 2 as measured by $AUC_{0-\infty}$, AUC_{0-6h}, or AUC_{last} for gemcitabine, dFdU and cisplatin-derived unbound platinum are summarized by cohort in Table 5. The 95% CI for each compound in cycle 2 overlapped the 95% CI in cycle 1, within and across each cohort. Exposure for each agent was fairly constant within a cohort and maintained its consistency across cohorts.

Anti-mapatumumab antibodies

One, previously untreated, patient in the 10 mg/kg cohort tested positive for anti-mapatumumab antibodies at day 29 of cycle 2. All mapatumumab plasma values observed for this subject were within the predicted concentration range.

Tumor response

Figure 3 shows reduction of target lesions in 26 of the 37 patients in whom target lesions were present. Confirmed partial responses were observed in 12 out of the 49 patients. Stable disease after 2 cycles was seen in 25 patients. Median duration of stable diseases and of partial responses was 6 months (range 1-33).

TRAIL-R1 expression

Tumor tissue was available in 35 patients. All tumors showed at least low cytoplasmic TRAIL-R1 expression in the majority of tumor cells. Staining intensity appeared not to be associated with tumor response (Table 6).

Table 5. Gemcitabine, dFdU and unbound platinum AUCs in the absence (cycle 1) and presence (cycle 2) of mapatumumab.

	Gemcitabine $AUC_{0-\infty}$ (µg·hour/mL)		dFdU AUC_{last} (µg·hour/mL)		Cisplatin-derived unbound platinum AUC_{0-6h} (µg·hour/mL)		Cisplatin-derived unbound platinum AUC_{last}(µg·hour/mL)	
	Cycle 1	Cycle 2	Cycle 1	Cycle 2	Cycle 1	Cycle 2	Cycle 1	Cycle 2
				1 mg/kg				
N	4	4	4	4	4	4	4	4
Mean	12.3	12.9	211	237	3.62	3.40	4.28	4.28
95% CI	9.80-14.8	9.78-16.1	179-242	182-292	2.82-4.42	1.97-4.84	2.81-5.76	2.01-6.55
				3 mg/kg				
N	7	7	7	7	7	7	7	7
Mean	11.4	11.8	206	199	3.28	3.02	3.84	3.54
95% CI	9.19-13.6	10.4-13.3	164-247	175-223	2.86-3.69	2.67-3.38	2.94-4.73	3.02-4.07
				10 mg/kg				
N	12	9	12	9	11	8	11	8
Mean	11.7	10.8	199	207	3.67	3.55	4.36	4.83
95% CI	10.4-13.1	8.2-13.3	177-220	189-225	3.23-4.11	2.97-4.13	3.61-5.11	3.89-5.77
				20 mg/kg				
N	13	9	13	9	13	9	13	9
Mean	11.4	10.7	194	194	3.71	3.69	4.59	4.76
95% CI	9.91-13.0	9.4-12.0	169-220	172-217	3.35-4.07	3.27-4.12	3.98-5.20	4.05-5.46
				30 mg/kg				
N	13	11	13	11	13	11	13	11
Mean	11.3	11.3	187	187	3.52	3.54	4.25	4.42
95% CI	9.82-12.7	10.2-12.4	168-206	170-205	3.34-3.70	3.21-3.87	3.89-4.61	3.85-4.99

In cycle 1, gemcitabine was administered immediately prior to cisplatin and 24 hours prior to the 1st mapatumumab dose. In the 2nd cycle (21 days later), gemcitabine, cisplatin, and mapatumumab were administered on the same day in immediate succession.

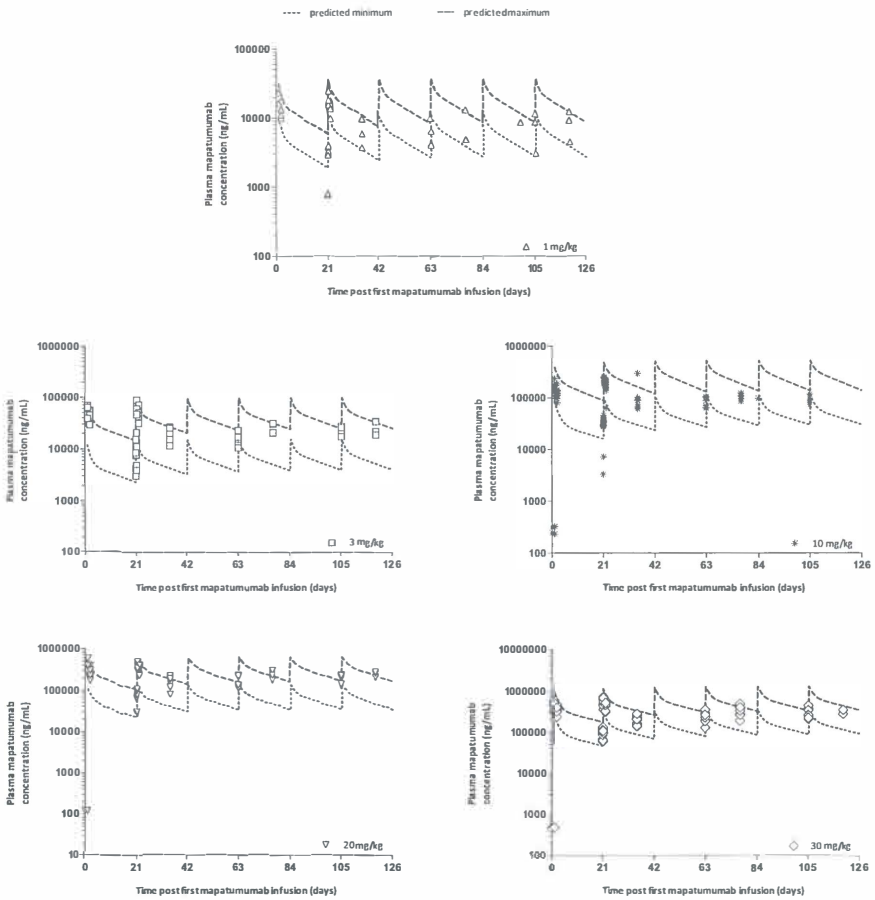


Figure 1. Plasma mapatumumab concentrations observed for individual subjects following 1, 3, 10, 20, or 30 mg/kg mapatumumab IV infusion doses given 21 days apart, with the expected minimum to maximum concentration range based on phase 1 study results.

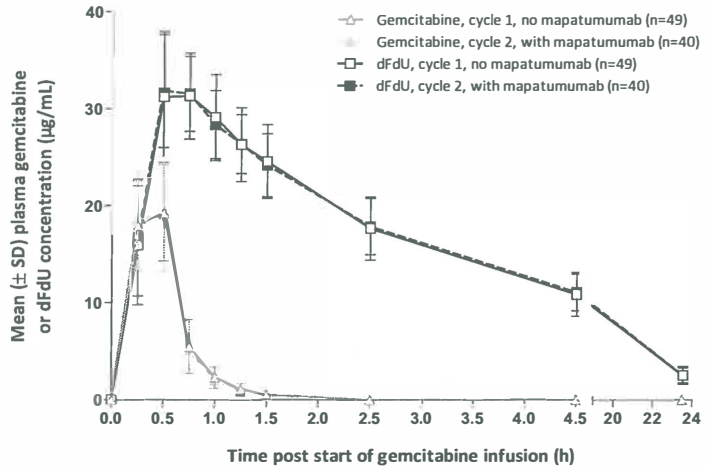


Figure 2A. Mean (± sd) plasma gemcitabine and dFdU concentrations following 1250 mg/m^2 IV gemcitabine doses administered as 30 minute infusion every 21 days (available data are presented for all cohorts combined).

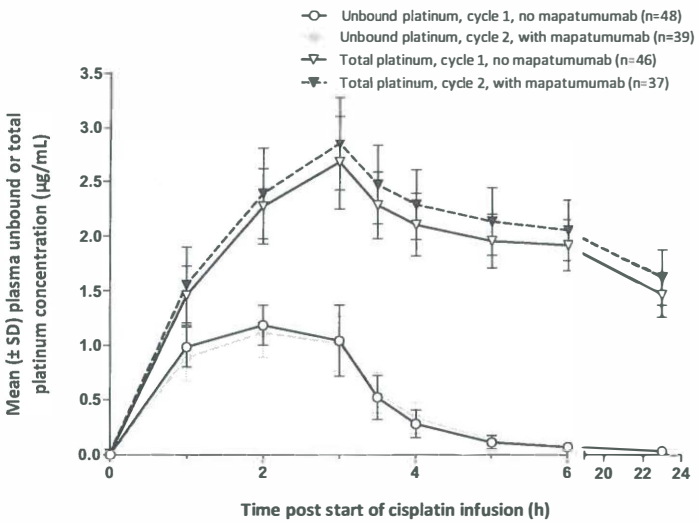


Figure 2B. Mean (± sd) plasma unbound and total platinum concentrations following 80 mg/m^2 IV cisplatin doses administered as 3 hour infusion every 21 days (available data are presented for all cohorts combined).

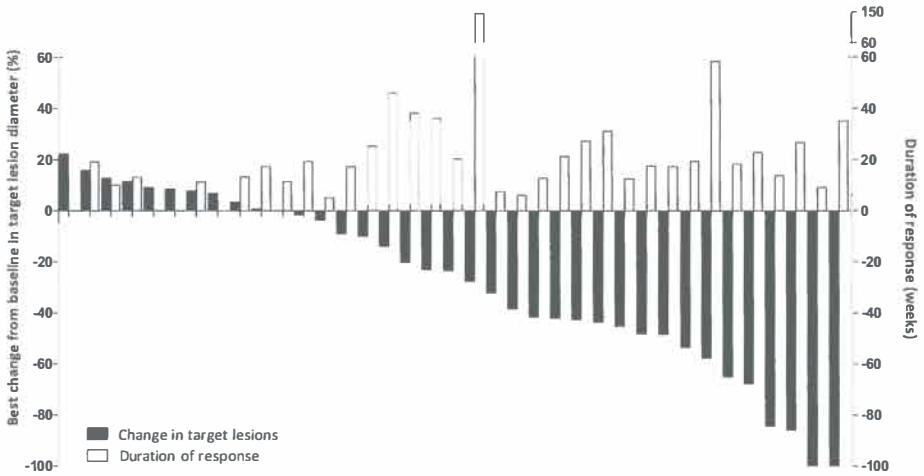


Figure 3. Maximum percent change in target lesions from baseline (black bars) and duration of response (transparent bars) per patient.

Table 6. Staining intensity in number of patients (n=35) in relation to tumor response .

Staining intensity	Tumor response according to RECIST[11]			
	PR	SD	PD	NA
No	0	0	0	0
Low	5	6	0	1
Moderate	1	4	0	3
Strong	3	7	4	1

Abbreviations: RECIST, Response Evaluation Criteria in Solid Tumors; PR, partial response; SD, stable disease; PD, progressive disease; NA, not available.

Discussion

This paper is the first full report on combining chemotherapy with a TRAIL-receptor targeting agent. In this study mapatumumab was combined with gemcitabine and cisplatin. This combination is safe and well tolerated. The side effects observed reflects the toxicity profile of the chemotherapeutic agents. There were no PK interactions observed between the study drugs. Partial responses as well as prolonged stable diseases were seen.

TRAIL-R1 is known to be expressed by human hepatocytes and therefore liver functions were closely monitored in this study (15). We observed transient and reversible grade 3 ALT and/or AST elevations in 8 patients. Single agent gemcitabine is known to induce transient grade 3-4 transaminase elevations in up to 25% of the patients (16-18). Nevertheless, a relation with mapatumumab cannot be completely ruled out. In a phase I study with single agent mapatumumab 2 patients at the highest dose level of 10 mg/kg every 14 days experienced DLTs consisting of grade 3 bilirubin and transaminase elevations that were considered to be probably related to mapatumumab (6). Other patients treated at 10 mg/kg experienced mild or moderate liver enzyme elevations, likely also partially related to liver metastases. However, in another phase I study with single agent mapatumumab, there was no evidence of hepatotoxicity (5).

Hematologic side effects in this study included neutropenia and thrombocytopenia. Grade 3 and 4 neutropenia was observed in 43% of the patients, with 1 patient experiencing neutropenic fever. Grade 3 and 4 thrombocytopenia occurred in 39% of the patients. These side effects, as well as the nausea and vomiting experienced by the majority of patients, were considered to be related to cisplatin and gemcitabine, and occurred in a frequency that is common for this combination (19,20). Other non-hematologic adverse events included fatigue, tinnitus and alopecia, and were mainly mild to moderate in severity.

Side effects did not influence the total amount of cisplatin and gemcitabine administered at the various dose levels of mapatumumab. As a result, the dose

intensity of cisplatin and gemcitabine was maintained throughout all mapatumumab dose levels explored.

The difficulty of assessing toxicity in studies evaluating a combination of various agents lies in the attribution of side effects. In the current study toxicity may have been the result of either 1 of the 3 drugs or of the combination of chemotherapy and mapatumumab. This dilemma is exemplified by the protocol amendments we had to make with regard to the DLT criteria. According to the initial criteria 4 out of 12 patients experienced a DLT at the 10 mg/kg dose level. One of these patients experienced a grade 3 hypokalemia due to cisplatin-induced vomiting; this is common and was unlikely to have been potentiated by mapatumumab. The protocol was amended during the 3rd cohort to consider only events related to mapatumumab or its interaction with gemcitabine and/or cisplatin as DLTs. According to the initial DLT criteria none of the patients at 1 mg/kg and 2 out of 7 patients at 3 mg/kg experienced DLTs. These 2 patients had liver enzyme elevations unrelated to mapatumumab, and 1 also experienced hyponatremia due to cisplatin-induced vomiting. Further dose escalation to 20 mg/kg was pursued without major toxicities, and at the highest tested dose level of 30 mg/kg no DLTs occurred.

Plasma mapatumumab concentrations were in agreement with the predicted exposure based on the previous phase I studies rendering it unlikely that co-administration of mapatumumab with gemcitabine and cisplatin has a meaningful impact on mapatumumab exposure (5;6). Furthermore, co-administration of mapatumumab did not affect exposure to gemcitabine, dFdU, or cisplatin.

In this study doses up to 30 mg/kg are evaluated, where the maximum dose given in the single agent phase I studies was 20 mg/kg (5). We considered the evaluation of 30 mg/kg safe, because administration of mapatumumab appeared to be well tolerated at dose levels up to and including 20 mg/kg in the (ongoing) phase I and II studies at that moment. In addition, the plasma mapatumumab concentrations in the subjects who received 20 mg/kg mapatumumab were consistent with the predicted exposures, and gemcitabine and cisplatin did not affect the mapatumumab plasma values.

Biological agents are often active at doses below the MTD, and the highest dose tested in clinical studies may exceed the effective dose by far. With lower mapatumumab doses maximum receptor occupation may have been reached already. In addition, there were no apparent differences in mapatumumab plasma concentrations between patients treated at 20 mg/kg compared to those receiving 30 mg/kg mapatumumab (Figure 1). Consequently, doses higher than 30 mg/kg mapatumumab are unlikely to yield higher plasma concentrations. However, in the absence of studies that determine the optimal biological dose, we recommend 30 mg/kg mapatumumab every 3 weeks for randomized studies evaluating the efficacy of gemcitabine, cisplatin and mapatumumab.

Most tumors in the present study did express TRAIL-R1. No association was found between baseline TRAIL-R1 expression and tumor response. It would be interesting to study TRAIL-R1 expression during therapy in relation to response since mapatumumab combined with cisplatin resulted in synergistic anti-tumor effects in vivo, possibly due to TRAIL-R1 upregulation by chemotherapy (21). In addition, it is not likely that TRAIL-R1 expression solely is predictive of response to mapatumumab-containing therapy, as several downstream factors in the TRAIL signaling pathway can affect the apoptotic response (22). This also points to the interest of combining mapatumumab with chemotherapy, as presumably optimal antitumor efficacy will be achieved by targeting more than one pathway (23). Moreover, the simultaneous engagement of both the intrinsic and extrinsic apoptotic pathway may result in the prevention of resistance to either drug. This is important because recently evidence has emerged that in TRAIL-resistant cells activation of the TRAIL receptors can lead to activation of nuclear factor-κB (NF-κB) which subsequently mediates proliferation, invasion and metastasis (24-26). To prevent such detrimental effects of TRAIL pathway directed therapy, the development of resistance to TRAIL receptor activating agents should be avoided. In tumor cell lines treatment with chemotherapy results in sensitization to TRAIL-mediated apoptosis, even in TRAIL resistant cell lines (27;28).

In this study, 26 out of the 37 patients with measurable disease showed a decrease in tumor lesions, and 12 patients achieved a partial response (Figure 3). These response

numbers are interesting compared to a historical overview of response rates in oncology phase I trials, including combination therapy studies (29). Moreover, stable disease was achieved in 51% of the patients and was markedly prolonged in a subset of patients, including several with tumor types that in general are marginally responsive to standard chemotherapy regimens such as biliary tract cancer and pancreatic cancer. However, the value of these findings is of course limited by the non randomized nature of the study. Furthermore, no patients were previously treated with gemcitabine and cisplatin. It is therefore difficult to assess the contribution of mapatumumab to the efficacy of this combination.

In conclusion, mapatumumab can be safely administered in combination with gemcitabine and cisplatin in doses up to 30 mg/kg. The pharmacokinetics of gemcitabine and cisplatin are not influenced by mapatumumab and vice versa. Responses and durable stable disease were seen across dose levels and in high numbers. Therefore, further studies, in a randomized setting, that explore the efficacy of this combination are warranted.

Acknowledgments

We thank Desiree van Boven-van Zomeren and Mei-Ho Lam for technical support. We thank Jos Bart, pathologist, for evaluating the immunohistochemical stainings.

Grant support

Human Genome Sciences Inc.

Disclosure of Potential Conflicts of Interest

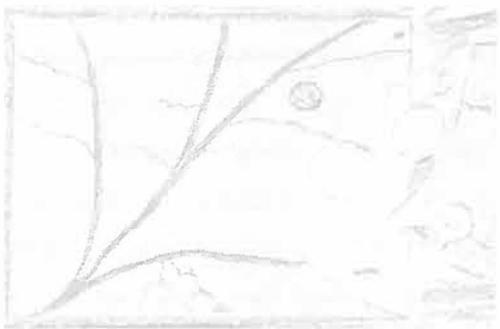
Norma Lynn Fox, employment and ownership, Human Genome Sciences. Renée Miceli, employment and ownership interest, Human Genome Sciences.

References

1. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305-8.
2. Ashkenazi A, Holland P, Eckhardt SG. Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). J Clin Oncol 2008;26:3621-30.
3. Pukac L, Kanakaraj P, Humphreys R, et al. HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types in vitro and in vivo. Br J Cancer 2005;92:1430-41.
4. Halpern W, Lincoln C, Sharifi A, et al. Variable distribution of TRAIL receptor 1 in primary human tumor and normal tissues. Eur.J.Cancer 2004 (Suppl 2; abstr 225).
5. Hotte SJ, Hirte HW, Chen EX, et al. A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies. Clin Cancer Res 2008;14:3450-5.
6. Tolcher AW, Mita M, Meropol NJ, et al. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. J Clin Oncol 2007;25:1390-5.
7. Greco FA, Bonomi P, Crawford J, et al. Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer. Lung Cancer 2008;61:82-90.
8. Kanzler S, Trarbach T, Heinemann V, et al. results of a phase 2 trial of HGS-ETR1 (agonistic human monoclonal antibody to TRAIL receptor 1) in subjects with relapsed or refractory colorectal cancer (CRC). Eur J Cancer 2005 (Suppl 3; abstr 630).
9. Younes A, Vose J, Zelenetz AD, et al. Results of a phase 2 trial of HGS-ETR1 (agonistic human monoclonal antibody to TRAIL receptor 1) in subjects with relapsed/refractory non-Hodgkin's lymphoma (NHL) (ETR1-HM01). Blood 2005:106 (abstr 489).
10. Humphreys R, Shepard L, Zhang Y-Q, et al. Novel, agonistic, human anti-TRAIL receptor monoclonal antibodies, HGS-ETR1 and HGS-ETR2, are capable of potently inducing tumor regression and growth inhibiton as single agents and in combination with chemotherapeutic agents in models of human NSCLC. Clin.Cancer Res 2003:9 (abstr B72).
11. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205-16.
12. Gietema JA, Hoekstra R, de Vos FY, et al. A phase I study assessing the safety and pharmacokinetics of the thrombospondin-1-mimetic angiogenesis inhibitor ABT-510 with gemcitabine and cisplatin in patients with solid tumors. Ann Oncol 2006;17:1320-7.
13. Gibaldi M, Perrier D. Pharmacokinetics. New York: Dekker; 1982.
14. Koornstra JJ, Kleibeuker JH, van Geelen CM, et al. Expression of TRAIL (TNF-related apoptosis-inducing ligand) and its receptors in normal colonic mucosa, adenomas, and carcinomas. J Pathol 2003;200:327-35.
15. Spierings DC, de Vries EG, Vellenga E, et al. Tissue distribution of the death ligand TRAIL and its receptors. J Histochem Cytochem 2004;52:821-31.
16. Fossella FV, Lippman SM, Shin DM, et al. Maximum-tolerated dose defined for single-agent gemcitabine: a phase I dose-escalation study in chemotherapy-naive patients with advanced non-small-cell lung cancer. J Clin Oncol 1997;15:310-6.
17. Possinger K, Kaufmann M, Coleman R, et al. Phase II study of gemcitabine as first-line chemotherapy in patients with advanced or metastatic breast cancer. Anticancer Drugs 1999;10:155-62.
18. Smorenburg CH, Bontenbal M, Seynaeve C, et al. Phase II study of weekly gemcitabine in patients with metastatic breast cancer relapsing or failing both an anthracycline and a taxane. Breast Cancer Res Treat 2001;66:83-7.
19. Sandler A, Ettinger DS. Gemcitabine: single-agent and combination therapy in non-small cell lung cancer. Oncologist 1999;4:241-51.

20. Sandler AB, Nemunaitis J, Denham C, et al. Phase III trial of gemcitabine plus cisplatin versus cisplatin alone in patients with locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 2000;18:122-30.
21. Kondo K, Yamasaki S, Sugie T, et al. Cisplatin-dependent upregulation of death receptors 4 and 5 augments induction of apoptosis by TNF-related apoptosis-inducing ligand against esophageal squamous cell carcinoma. Int J Cancer 2006;118:230-42.
22. Carlo-Stella C, Lavazza C, Locatelli A, Vigano L, Gianni AM, Gianni L. Targeting TRAIL agonistic receptors for cancer therapy. Clin Cancer Res 2007;13:2313-7.
23. de Vries EG, de Jong S. Exploiting the apoptotic route for cancer treatment: a single hit will rarely result in a home run. J Clin Oncol 2008;26:5151-3.
24. Baader E, Toloczko A, Fuchs U, et al. Tumor necrosis factor-related apoptosis-inducing ligand-mediated proliferation of tumor cells with receptor-proximal apoptosis defects. Cancer Res 2005;65:7888-95.
25. Belyanskaya LL, Ziogas A, Hopkins-Donaldson S, et al. TRAIL-induced survival and proliferation of SCLC cells is mediated by ERK and dependent on TRAIL-R2/DR5 expression in the absence of caspase-8. Lung Cancer 2008;60:355-65.
26. Trauzold A, Siegmund D, Schniewind B, et al. TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. Oncogene 2006;25:7434-9.
27. Galligan L, Longley DB, McEwan M, Wilson TR, McLaughlin K, Johnston PG. Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. Mol Cancer Ther 2005;4:2026-36.
28. Lacour S, Hammann A, Wotawa A, Corcos L, Solary E, Dimanche-Boitrel MT. Anticancer agents sensitize tumor cells to tumor necrosis factor-related apoptosis-inducing ligand-mediated caspase-8 activation and apoptosis. Cancer Res 2001;61:1645-51.
29. Horstmann E, McCabe MS, Grochow L, et al. Risks and benefits of phase 1 oncology trials, 1991 through 2002. N Engl J Med 2005;352:895-904.

CHAPTER 4



A phase Ib study of the VEGF receptor tyrosine kinase inhibitor tivozanib and FOLFOX6 in patients with advanced gastrointestinal malignancies: a preliminary thesis report

CNAM Oldenhuis[1], A van der Gaast[2], M Cotreau[3], MW den Hollander[1], WJ Loos[2], L van Doorn[2], J Jac[3], B Esteves[3], EGE de Vries[1], JA Gietema[1], FALM Eskens[2].

[1]Department of Medical Oncology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; [2]Department of Medical Oncology, Erasmus University Medical Center Rotterdam, Daniel den Hoed Cancer Center, Rotterdam, The Netherlands; [3]AVEO Pharmaceuticals, Cambridge, MA, USA.

Note: Presented in part at the EORTC-NCI-AACR International Symposium on Molecular Targets and Cancer Therapeutics, Berlin, Germany, Nov 16-19, 2010 and at the American Society of Clinical Oncology (ASCO) Gastrointestinal Cancers symposium, San Francisco, CA, Jan 20-22, 2011.

Abstract

Purpopse: Tivozanib is a highly potent and selective tyrosine kinase inhibitor of all three VEGF receptors with additive antitumor activity to 5-fluorouracil (5FU) in preclinical models. In phase I and II studies, tiovozanib monotherapy showed anti-tumor activity. This phase 1b study was conducted to determine the maximum tolerated dose (MTD), dose-limiting toxicities (DLTs), pharmacokinetics (PK) and anti-tumor activity of escalating doses of tivozanib and standard dose leucovorin, 5FU, and oxaliplatin (FOLFOX6) in patients with advanced gastrointestinal tumors.

Patients and methods: Patients with advanced gastrointestinal malignancies were eligible. Tivozanib was administered orally once daily for 21 days in 28-day cycles, with FOLFOX6 administered every 14 days. Blood for tivozanib, 5FU and oxaliplatin PK was sampled during the first cycle. Toxicity was scored according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE), version 3.0. Patients were allowed to continue tivozanib following discontinuation of FOLFOX6. Tumor response was assessed after every 2nd cycle by RECIST version 1.0.

Results: So far, 30 patients, (18 male, 12 female, median age 58 years years (range, 40-75) were assigned to tivozanib 0.5 mg (n=9), 1.0 mg (n=3) and 1.5 mg (n=18). DLTs consisted of one episode of reversible grade 4 AST and ALT elevation and one episode of reversible grade 3 diarrhea at tivozanib 0.5 mg. No DLTs occurred at tivozanib 1.0 mg. At tivozanib 1.5 mg DLT consisted of one episode of grade 3 vertigo and one episode of grade 3 epileptic seizure during cycle 2. Other grade 3/4 adverse events (AEs) included hypertension (n = 11) , neutropenia (n = 6) and fatigue (n = 4). No PK interactions were observed. Of the 30 patients, 1 patient had a complete response, 8 patients achieved a partial response and 12 experienced stable disease. Five patients had progressive disease and 5 patients were not evaluable for response.

Conclusions: The combination of tivozanib and FOLFOX6 is feasible and safe with drug related AEs that did not appear to be more frequent or severe than known to occur with either FOLFOX6 or tivozanib alone The recommended dose for future combination studies is 1.5 mg/day. Observed clinical activity merits further exploration in several gastrointestinal tumors.

Introduction

Angiogenesis, the formation of new blood vessels, is essential for tumor growth. Vascular Endothelial Growth Factor (VEGF) is the most prominent and potent proangiogenic factor. VEGF binds with high affinity to the endothelial VEGF receptors (VEGFRs) and this results in endothelial cell proliferation, cell survival and angiogenesis. Various tumors express high VEGF levels and this has been found to be correlated with poor clinical outcome (1).

Tivozanib (AV-951, KRN951) is a potent and selective oral VEGFR1, -2 and -3 tyrosine kinase inhibitor active at subnanomolar concentrations with half maximal inhibitory concentrations (IC50) of 0.21, 0.16, and 0.24 nM, respectively (2). In preclinical models, tivozanib demonstrated profound anti-angiogenesis and anti-tumor activity. In a single-agent phase I study, 41 patients with advanced solid tumors were treated with tivozanib for 28 days followed by a 2 week rest period (3). The maximum tolerated dose (MTD) in this study was 1.5 mg with controllable hypertension as the most common, dose-related adverse event. Its serum half-life was 4.7 days, whereas pharmacodynamic analysis revealed dose dependent changes in serum concentrations of both VEGF and sVEGFR2. Tumor shrinkage was observed in 35% of the patients. In a phase II randomized discontinuation study with single agent tivozanib, 272 patients with advanced renal cell carcinoma were treated (4). The overall response rate was 30% and a median progression-free survival of 11.7 months was observed. Hypertension and dysphonia were the most frequent drug-related adverse events (AE) in this study. A phase III trial comparing tivozanib with sorafenib in advanced renal cell cancer has completed patient enrollment (NCT01030783).

In the single agent phase 1 study, durable stable disease was observed among patients with gastrointestinal malignancies. In addition, the combination of tivozanib with fluorouracil (5FU) and other cytotoxic agents resulted in additional anti-tumor activity in the MX-1 breast cancer xenograft model (5). We therefore performed a phase Ib study to evaluate safety, tolerability and maximum tolerated dose of tivozanib in combination with FOLFOX6 (leucovorin, 5FU, and oxaliplatin) in patients with advanced

gastrointestinal malignancies. Secondary objectives were to determine the pharmacokinetic profile of tivozanib and its influence on 5FU and oxaliplatin concentrations and to assess the antitumor activity of this combination.

Patients and methods

Eligibility criteria

Patients with a histologically or cytologically confirmed, metastatic gastrointestinal malignancy for whom FOLFOX6 was considered an appropriate treatment were eligible. Other inclusion criteria included: documented progressive disease; age ≥ 18 years; measurable or evaluable disease by RECIST (6); no more than 2 prior chemotherapy regimens for metastatic disease; at least 3 weeks since prior systemic anticancer treatment; resolution of toxicities from prior treatment to ≤ grade 1; Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2 and a life expectancy ≥ 3 months. Key exclusion criteria included: central nervous system metastases; second malignancies; inadequate hematologic, hepatic and renal function; significant cardiovascular disease; active infection; surgery in the previous 6 weeks; unhealed wounds, ulcers or bone fractures; bleeding disorders; deep venous thrombosis, pulmonary embolus or cerebrovascular accident within 12 months prior to the start of treatment; need for anticoagulation treatment or local radiotherapy within 2 weeks before the start of cycle 1.

The local Ethics Committees approved the study. Written informed consent was obtained before study-related procedures were started. The study is registered under trial number NCT00660153.

Study design

A cycle was defined as 28 days. Patients received tivozanib orally at escalating dose levels in capsules of 0.5, 1.0 or 1.5 mg once daily for 3 weeks followed by 1 week off drug. Based on the phase 1 study, where 2.0 mg/day exceeded the MTD, 1.5 mg per day was the maximum dose that would be tested (3). FOLFOX6 was administered on

days 1 and 15 of a 28 day cycle. FOLFOX6 consisted of leucovorin 400 mg/m^2 as a 2-hour infusion, and concurrently given oxaliplatin 85 mg/m^2 intravenously (iv) over 2 hours, followed by bolus 5FU 400 mg/m^2 iv over 10 minutes and a 46-hour continuous infusion 5FU 2400 mg/m^2. On days when both tivozanib and FOLFOX6 were co-administered, tivozanib was taken 5 minutes prior to the start of FOLFOX6. Dose reductions were allowed for patients with ≥ grade 3 adverse events related to tivozanib, except for hypertension. Hypertension was treated according to local protocols before dose reduction was considered. Tivozanib could be reduced in steps of 0.5 mg, to a minimum dose of 0.5 mg daily.

A standard 3+3 phase I trial design was used (7). Three patients were enrolled at each dose level. If 1 of 3 patients experienced dose-limiting toxicity (DLT) during cycle 1, the cohort was expanded to 6 evaluable patients. If a DLT was observed in ≥ 2 out of 6 patients in a cohort the MTD was exceeded. If 0 of 3 or 1 of 6 patients experienced a DLT during cycle 1, dose escalation was pursued. Dose limiting toxicity was defined as: Any grade 4 nonhematological toxicity; neutropenia ≥ grade 3 associated with fever or sepsis; grade 4 neutropenia during 5 days or longer; grade 4 thrombocytopenia or bleeding requiring transfusion of platelets; toxicity of any grade resulting in inability to complete cycle 1 or resulting in interruption of treatment for > 2 weeks and any grade 3 nonhematologic toxicity lasting > 3 days despite optimal supportive care with the exception of alopecia of any grade, grade 3 controllable hypertension, grade 3 self-limiting or medically controllable toxicity, grade 3 AST or ALT lasting ≤ 1 week, grade 3 oxaliplatin-induced neurotoxicity in patients with prior exposure to oxaliplatin, grade 3 gastrointestinal toxicity lasting ≤ 1 week. NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 was used to grade toxicities (8). Once the MTD was reached, 12 additional patients with measurable disease could be enrolled at that dose.

Patients could receive combined treatment for up to 1 year. If FOLFOX6 was discontinued earlier due to unacceptable toxicity in the absence of disease progression, tivozanib monotherapy could be continued. Patients were withdrawn

from the study in case of disease progression, unacceptable toxicity or refusal of treatment. Response was assessed after every 2nd cycle using RECIST version 1.0 (6).

Pharmacokinetic (PK)studies

Patients received a single dose tivozanib at day -5 (± 2 days). PK sampling was performed pre-dose and 1, 2, 4, 8 and 24 hours following dosing. On days 1 and 15 in cycle 1, PK samples for determination of serum levels tivozanib and plasma concentrations 5FU and unbound platinum were taken pre-dose, 5 minutes prior to the end of oxaliplatin bolus, immediately after 5FU bolus, 1 hour after start of the continuous 5FU infusion, at 24 hours and 48 hours following the start of FOLFOX6 (prior to tivozanib dosing), and 5 minutes before the end of continuous 5FU infusion. In addition, blood samples were collected on days 8, 21 (pre-dose and 1, 2, 4, 8 and 24 hours following dosing) and 22 of cycle 1 and on day 1 of cycle 2. Serum tivozanib samples were stored at –20°C until analysis by a validated reversed-phase liquid chromatography tandem mass spectrometry (LC-MS/MS) method in the range of 0.100 to 100 ng/mL, with KRN633 as internal standard.

For 5FU analysis, 30 µL aliquots of human lithium heparinized plasma, or appropriate dilutions of the sample, were extracted after the addition of 10 µL internal standard (either 5-chlorouracil or 13C/15N2 labeled 5-fluorouracil) with 1.5 mL ethyl acetate. After evaporation of the organic phase and re-suspension of the residue in an aliquot of 100 µL of Ringer's solution, an aliquot of 50 µL has been injected into the LC-MS/MS system as described peviously (9).

Plasma concentrations of unbound oxaliplatin-derived platinum were determined as described for cisplatin-derived platinum (10). In brief 500 µL aliquots of the plasma supernatant were mixed with 1.0-mL aliquots of ice-cold (-20°C) ethanol. Ethanolic supernatant was collected by centrifugation at 18,000 x g for 5 min after which the clear supernatant was stored at T < -70°C until analysis. On the day of analysis aliquots of 1000 µL of the ethanolic supernatant, or dilutions of the supernatant in blank ethanolic supernatant, were evaporated to dryness under nitrogen at T = 80°C, and the residue reconstituted in 200 µL diluent (i.e., water containing 0.2% (v/v) Triton X-100

and 0.06% (w/v) cesium chloride), from which subsequently aliquots of 20 µL, in duplicate, were injected onto the graphite furnace of a Perkin Elmer Model 4110 ZL atomic absorption spectrophotometer (Uberlingen, Germany). Platinum peak areas were measured at 265.9 nm with a lower limit of quantitation of 0.0300 µg/mL.

Results

General

The results presented in this report are preliminary en incorporate data until November 1st 2011. Overall, 30 patients were enrolled (Table 1). Patient characteristics are shown in Table 2. These patients received 113 cycles of combination therapy (median 4, range 1-8). In addition, 13 patients received 103 cycles tivozanib monotherapy (median 6, range 1-20). Three patients are continuing to receive treatment.

Table 1. Dose levels.

Cohort	Tivozanib	FOLFOX6	No. of patients
1	0.5 mg/day	Standard	9
2	1.0 mg/day	Standard	3
3	1.5 mg/day	Standard	6
MTD expansion	1.5 mg/day	Standard	12

MTD, maximum tolerated dose.

Two patients in the first cohort received a higher than per protocol dose 5FU. The first patient revealed a grade 2 leucopenia and the second patient suffered from reversible, grade 3 diarrhea. Treatment was stopped and both patients were replaced. The third patient in the first cohort was replaced because of early progressive disease, which manifested during the first cycle.

Safety and tolerability

Tivozanib in combination with FOLFOX6 was in general well tolerated. Most common observed AEs were fatigue, peripheral sensory neuropathy and anemia (Table 3).

Grade 3 or 4 AEs were hypertension (n = 11), neutropenia (n = 6) and fatigue (n = 4). Hematologic toxicity and neuropathy were considered most likely related to FOLFOX6. Hypertension was considered most likely related to tivozanib. Hypertension was well controlled with antihypertensive medications in all patients. Neutropenia was not associated with fever. Neutropenia, thrombocytopenia and peripheral sensory neuropathy resulted in dose reductions of oxaliplatin and/or 5FU and FOLFOX6 treatment delays across tivozanib dose levels in 18 patients (60%).

Table 2. Baseline patient characteristics.

Characteristic	N = 30
Median age (range), y	58 (40-75)
Male sex, n (%)	18 (60)
Tumor type, n (%)	
Gastric/esophageal adenocarcinoma	13 (43)
Colorectal carcinoma	6 (20)
Pancreatic adenocarcinoma	10 (33)
Small bowel adenocarcinoma	1 (3)

DLT

In the 0.5 mg cohort, 1 patient experienced grade 4 AST and grade 3 ALT elevations on day 2 of the first cycle. All study medication was stopped, after which AST and ALT normalized. FOLFOX6 was restarted with 1 week delay and again resulted in reversible grade 4 AST and ALT elevations. This toxicity was regarded as definitively related to 5FU and oxaliplatin and possibly related to tivozanib. Reversible grade 3 diarrhea was observed in another patient in this cohort. This patient received a higher than per protocol dose 5FU and was considered not evaluable. The patient was replaced and dose escalation was pursued. In the 1.0 mg cohort no DLTs occurred.

At 1.5 mg/day tivozanib one patient experienced reversible grade 3 dizziness during cycle 1. Another patient suffered from a grade 3 epileptic seizure. However, this event occurred in cycle 2 and was therefore not considered a protocol-defined DLT. No other

DLTs were observed in the first 6 patients at 1.5 mg tivozanib and therefore 12 additional patients were enrolled. No DLTs occurred in this expansion cohort.

Table 3. Observed adverse events (≥15% of the patients).

Adverse event	All grades (n = 30)		Grade 3 / 4 (n = 30)	
	n	%	n	%
Fatigue	27	90	4	13
Peripheral sensory neuropathy	25	83	0	0
Anemia	24	70	0	0
Nausea	21	70	0	0
AST elevation	21	70	1	3
Thrombocytopenia	20	67	1	3
ALT elevation	19	63	1	3
Leucopenia	19	63	4	13
ALP elevation	18	60	0	0
Diarrhea	17	57	2	7
Hypertension	16	53	11	37
Vomiting	16	53	0	0
Neutropenia	15	50	6	20
Stomatitis	15	50	0	0
Hoarseness	11	37	0	0
Proteinuria	6	20	0	0

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

Pharmacokinetics

Results are available for the first 22 patients enrolled. The mean tivozanib serum concentrations at steady state do not appear to be influenced by FOLFOX6 treatment and were comparable to levels observed in the tivozanib monotherapy studies (Figure 1A). Unbound platinum and 5FU plasma concentrations were similar on days 1 and 15, indicating that increasing serum levels of tivozanib did not influence plasma concentrations of unbound platinum or 5FU (Figures 1B and 1C).

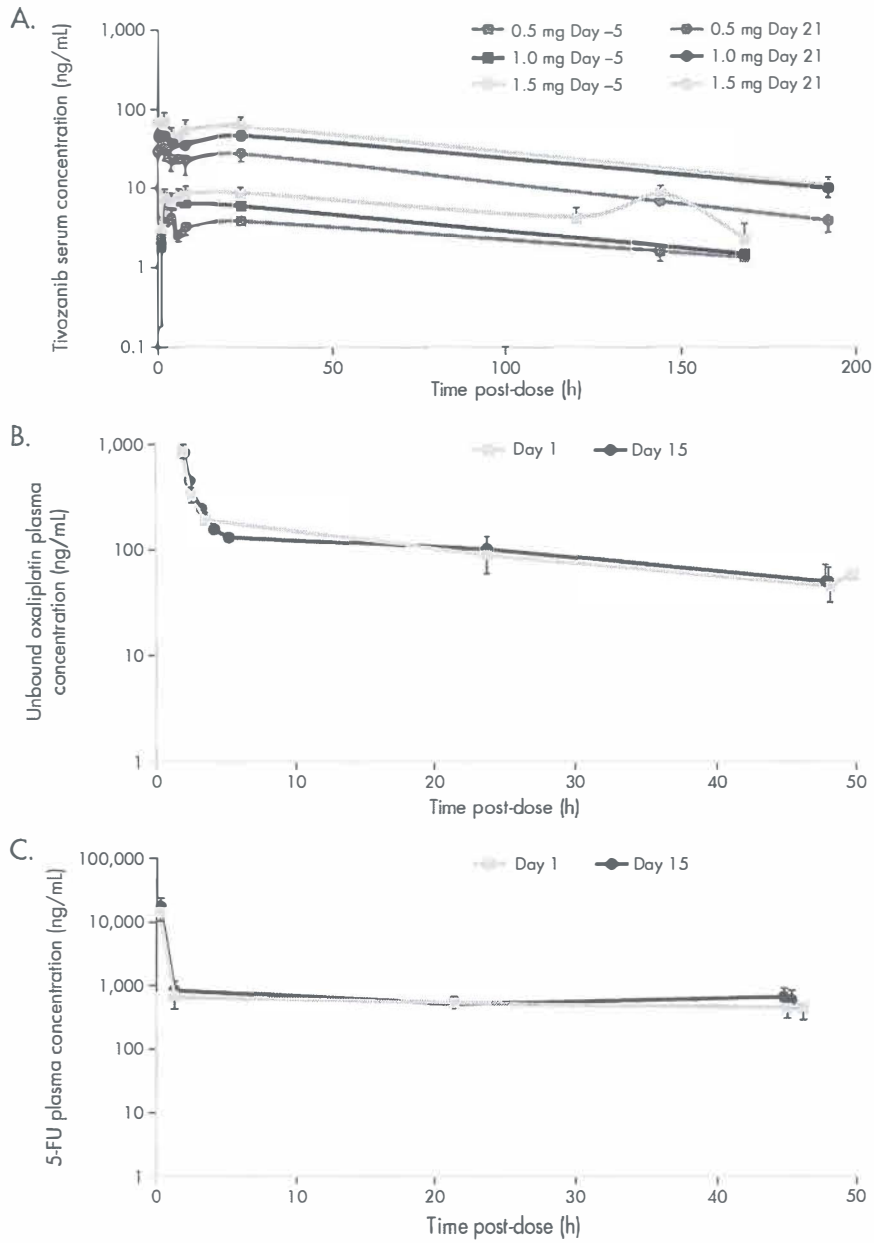


Figure 1. Concentration-time profiles depicting mean values (± SEM) for (A) serum tivozanib concentration, (B) unbound oxaliplatin plasma concentration and (C) 5-FU plasma concentration as a function of time post-dose. 5-FU, 5-fluorouracil; SEM, standard error of the mean.

Antitumor activity

Of the 30 patients, 1 patient with metastatic duodenal carcinoma had a confirmed complete response, currently lasting 36 weeks, and 8 patients obtained a partial response. Another 12 patients experienced stable disease. Responses occurred in patients with oesophageal cancer (n = 4), pancreatic cancer (n = 2), colorectal cancer (n = 1), gastric cancer (n = 1) and small bowel cancer (n = 1).

The median duration of treatment was 5 cycles (range 1-27+ cycles). Percentage change of target lesions was available for 15 patients. Thirteen out of these 15 patients showed shrinkage of the target lesions (Figure 2). Seven out of the 9 patients (78%) with a partial or complete response developed hypertension during the study versus 9 out of the remaining 21 patients (43%, *P* = .118; Fisher's exact test).

Figure 2. Waterfall plot of maximum tumor change from baseline for the 15 patients currently available per tivozanib dose. * Indicates patients who are still receiving treatment.

Discussion

This paper is a preliminary report of an ongoing phase I study combining the novel, oral angiogenesis inhibitor tivozanib with FOLFOX6 chemotherapy in patients with gastrointestinal tumors. The combination was well tolerated. The observed AEs predominantly consisted of well known FOLFOX6-related toxicity and tivozanib induced, controllable hypertension. One complete response and partial responses were observed throughout the cohorts.

The MTD of tivozanib in combination with standard FOLFOX6 was not reached at 1.5 mg/day. We did not explore higher doses, as the MTD in the single agent tivozanib phase I study was exceeded when using the next dose step of 2.0 mg/day (3). Tumor responses were observed in that single agent study also on dose levels <2.0 mg/day. Therefore, 1.5 mg/day is considered to be an effective biological dose and the recommended phase II dose for further studies combining FOLFOX6 and tivozanib.

The most common observed AEs in this study were fatigue, peripheral sensory neuropathy, anemia and nausea. Incidences of neuropathy and nausea were comparable to those seen with FOLFOX6 alone and are therefore considered most likely related to the chemotherapeutic agents (11,12). Fatigue may be chemotherapy, tivozanib or cancer related. The incidence of fatigue of any grade in this study was with 90% remarkably higher compared to the 51% incidence rate in the single agent tivozanib phase I study (3). We consider this difference to be the consequence of the combination therapy.

Thrombocytopenia and neutropenia were seen in respectively 67% and 50% of the patients during treatment with FOLFOX6. Neutropenic episodes were not associated with fever. However, thrombocytopenia and neutropenia resulted in delayed dosing and dose reductions in 18 patients. Moreover, several patients could not continue FOLFOX6 due to delayed recovery. In the final analysis of this study we will report if dose intensity was stable and sufficient throughout the cohorts.

Hypertension is a well-known side effect of angiogenesis targeting agents and blood pressure was therefore carefully monitored in this study (13). In 53% of all patients hypertension developed, and 37% required antihypertensive treatment. Fortunately,

in all patients in whom treatment had to be started, hypertension turned out to be easily manageable and in no patient resulted in interruption or termination of study drug administration. In the single agent studies frequencies of 45-58% (all grades) were reported (3,4). Frequencies for other VEGF tyrosine kinase inhibitors vary from 1-40% (14).

The observed toxicity in our study was comparable to that seen in other studies combining a pan-VEGF-R tyrosine kinase inhibitor with FOLFOX chemotherapy (15-17).

Hypertension is probably associated with improved clinical outcome in patients treated with VEGF-R tyrosine kinase inhibitors (18,19). Although not statistically significant, there was a trend toward a higher incidence of hypertension in the patients with a tumor response compared to the remaining patients in our study (78% versus 43%).

Serum tivozanib levels in this combination study were comparable to those observed in the single agent phase I study (3). Therefore, serum tivozanib levels seem not to be influenced by FOLFOX6 chemotherapy. Furthermore, plasma levels of unbound platinum and 5FU were not affected by increasing tivozanib levels during the treatment cycle.

As a result of excessive production of proangiogenic factors and downregulation of antiangiogenic molecules, structural abnormalities make tumor vessels hyperpermeable. VEGF-R targeting agents like tivozanib can potentially restore the disbalance between pro- and antiangiogenic factors and result in tumor vessel normalization, as was shown for this drug in several preclinical studies (20-21). As a consequence, interstitial fluid pressure may decrease and tumor perfusion may improve, making the tumor more accessible for cytotoxic chemotherapy. In theory, this might increase tumor response rates, without effecting chemotherapy induced toxicity. Efficacy in our study was indeed promising, with a decrease in tumor size in 13 of the 15 patients (87%) with measurable lesions. One patient achieved a complete response and 8 patients a partial response. Furthermore, keeping in mind that documented progressive disease was one of the inclusion criteria for this study, it is

also noteworthy to mention that 6 patients, having stable disease as best response, were on treatment for ≥ 6 cycles.

In conclusion, the combination of tivozanib and FOLFOX6 is feasible and safe. The recommended tivozanib dose for future combination studies with FOLFOX6 is 1.5 mg/day. Observed clinical activity merits further exploration in several gastrointestinal tumors.

References

1. Schoenleber SJ, Kurtz DM, Talwalkar JA, et al: Prognostic role of vascular endothelial growth factor in hepatocellular carcinoma: systematic review and meta-analysis. Br J Cancer 100:1385-92, 2009
2. Nakamura K, Taquchi E, Miura T, et al: KRN951, a highly potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, has antitumor activities and affects functional vascular properties. Cancer Res 66:9134-42, 2006
3. Eskens FA, de Jonge MJ, Bhargava P, et al: Biologic and clinical activity of tivozanib (AV-951, KRN-951), a selective inhibitor of VEGF receptor-1, -2, and –3 tyrosins kinases, in a 4-week-on, 2-week-off schedule in patients with advanced solid tumors. Clin Cancer Res 17:7156–63, 2011
4. Nosov D, Bhargava P, Esteves WB, et al: Final analysis of the phase II randomized discontinuation trial (RDT) of tivozanib (AV-951) versus placebo in patients with renal cell carcinoma (RCC). J Clin Oncol 29:2011 (suppl; abstr 4550)
5. Lin J, Jiang F, Shen L, et al: Tivozanib activity in combination with capecitabine, 5-fluorouracil (5-FU) or docetaxel in traditional or engineered subcutaneous breast tumor models. EORTC-NCI-AACR International Symposium on Molecular Targets and Cancer Therapeutics; 2010, abstr 90
6. Therasse P, Arbuck SG, Eisenhauer EA, et al: New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92:205-16, 2000
7. Eisenhauer EA, O'Dwyer PJ, Christian M, et al: Phase I clinical trial design in cancer drug development. J Clin Oncol 18:684-92, 2000
8. http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf
9. Konings IR, Sleijfer S, Mathijssen RH, et al: Increasing tumoral 5-fluorouracil concentrations during a 5-day continuous infusion: a microdialysis study. Cancer Chemother Pharmacol 67:1055-62, 2011
10. Loos WJ, de Jongh FE, Sparreboom A, et al: Evaluation of an alternate dosing strategy for cisplatin in patients with extreme body surface area values. J Clin Oncol 24:1499-506, 2006
11. Maindrault-Goebel F, Louvet C, André T, et al: Oxaliplatin added to the simplified bimonthly leucovorin and 5-fluorouracil regimen as second-line therapy for metastatic colorectal cancer (FOLFOX6). Eur J Cancer 35:1338-42, 1999
12. Allegra CJ, Yothers G, O'Connell MJ, et al: Initial safety report of NSABP C-08: A randomized phase III study of modified FOLFOX6 with or without bevacizumab for the adjuvant treatment of patients with stage II or III colon cancer. J Clin Oncol 27:3385-90, 2009
13. Maitland ML, Bakris GL, Black HR, et al: Initial assessment, surveillance, and management of blood pressure in patients receiving vascular endothelial growth factor signaling pathway inhibitors. J Natl Cancer Inst 102:596-604, 2010
14. Escalante CP and Zalpour A. Vascular endothelial growth factor inhibitor-induced hypertension: basics for primary care providers. Cardiol Res Pract 816897, 2011
15. Chen E, Jonker D, Gauthier I, et al: Phase I study of cediranib in combination with oxaliplatin and infusional 5-Fluorouracil in patients with advanced colorectal cancer. Clin Cancer Res 15:1481-6, 2009
16. Thomas AL, Trarbach T, Bartel C, et al: A phase IB, open-label dose-escalating study of the oral angiogenesis inhibitor PTK787/ZK 222584 (PTK/ZK), in combination with FOLFOX4 chemotherapy in patients with advanced colorectal cancer. Ann Oncol 18:782-8, 2007
17. Sharma S, Abhyankar V, Burgess RE, et al: A phase I study of axitinib (AG-013736) in combination with bevacizumab plus chemotherapy or chemotherapy alone in patients with metastatic colorectal cancer and other solid tumors. Ann Oncol 21:297-304, 2010
18. Rini BI, Cohen DP, Lu DR, et al: Hypertension as a biomarker of efficacy in patients with metastatic renal cell carcinoma treated with sunitinib. J Natl Cancer Inst 103:763-73, 2011
19. Bhargava P, Esteves B, Al-Adhami M, et al: Effect of hypertension, nephrectomy, and prior treatment on the efficacy of tivozanib (AV-951) in a phase II randomized discontinuation trial (RDT) in patients with renal cell carcinoma (RCC). ASCO Genitourinary Cancers Symposium; 2010, abstr 342

20. Kamoun WS, Ley CD, Farrar CT, et al: Edema control by cediranib, a vascular endothelial growth factor receptor-targeted kinase inhibitor, prolongs survival despite persistent brain tumor growth in mice. J Clin Oncol 27:2542-52, 2009

21. Dickson PV, Hamner JB, Sims TL et al: Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenografts results in improved delivery and efficacy of systemically administered chemotherapy. Clin Cancer Res 13:2942-50, 2007

CHAPTER 5

Prognostic versus predictive value of biomarkers in oncology

CNAM Oldenhuis, SF Oosting, JA Gietema, EGE de Vries.

Department of Medical Oncology. University Medical Center Groningen, The Netherlands.

European Journal of Cancer. 2008;44:946-53.

Abstract

Numerous options are currently available for tumour typing. This has raised intense interest in the elucidation of prognostic and predictive markers. A prognostic biomarker provides information about the patients overall cancer outcome, regardless of therapy, while a predictive biomarker gives information about the effect of a therapeutic intervention. A predictive biomarker can be a target for therapy. Among the genes that have proven to be of relevance are well-known markers such as ER, PR and HER2/neu in breast cancer, BCR-ABL fusion protein in chronic myeloid leukaemia, *c-KIT* mutations in GIST tumours and *EGFR1* mutations in NSCLC. Several reasons for the difficult elucidation of new markers will be addressed including the involvement of cellular pathways in tumour biology instead of single genes and interference in disease outcome as a result of anticancer therapies. Future perspectives for the development of prognostic and predictive markers will be given.

Introduction

With the availability and application of various treatment modalities, survival among cancer patients has improved over the past decades. However, there are still many patients who receive anticancer therapy from which they do not benefit while they do experience toxicity. In recent years, a widespread search for new, tumour biology driven therapeutics has started. This has raised intense interest in the elucidation of corresponding prognostic and predictive biomarkers in order to improve outcome by better patient selection for an anticancer treatment. A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacological responses to a specified therapeutic intervention (1). Biomarkers can be determined in numerous ways, for example, in easy obtainable body fluids serving as surrogate biological assay, like plasma, serum or urine. But also more invasive techniques requiring tumour tissue for immunohistochemistry as well as DNA and RNA analyses are widely used. A prognostic biomarker provides information about the patients overall cancer outcome, regardless of therapy. The presence or the absence of such a prognostic marker can be useful for the selection of patients for a certain treatment, but does not predict the response to this treatment. Prognostic biomarkers can be separated in two groups: biomarkers that give information on recurrence in patients who receive curative treatment and biomarkers that correlate with the duration of (progression free) survival in patients with metastatic disease. According to a NIH Consensus Conference, a clinical useful prognostic marker must be a proven independent, significant factor, that is easy to determine and interpret and has therapeutic consequences (2). A biomarker with predictive value gives information on the effect of a therapeutic intervention in a patient. A predictive biomarker can also be a target for therapy. One can distinguish upfront and early predictive markers. The first can be used for patient selection and the second provides information early during therapy.

The current interest in marker determination is boosted by the discovery of genes that have proven to be of clinical relevance such as the oestrogen receptor (ER), the progesterone receptor (PR) and HER2/neu in breast cancer, BCR-ABL fusion protein in chronic myeloid leukaemia, *c-KIT* mutations in gastrointestinal stromal tumours (GIST) and *epidermal growth factor receptor 1* (*EGFR1*) mutations in non-small cell lung cancer (NSCLC). These genes all seem to be key regulators of development, growth and proliferation in the respective tumour types. Euphoria is now somewhat tempered because the discovery of other so-called promising markers translates rather slowly into clinical applicability. One reason for this is the fact that the course of most of malignancies is the consequence of a number of essential alterations in tumour cells rather than a single mutation (3). In addition, the limited size of most studies and variable techniques used for marker determination plays a role. Often initially reported promising results are not reproducible. In an attempt to optimise biomarker studies, Hayes and colleagues proposed a tumour marker utility grading system (TMUGS). For each biomarker a grade of utility is assigned, accompanied by a level of evidence (LOE) that scores the quality of the research. The LOE categories range from I to V. Level V evidence is obtained from case reports and clinical experience and is considered weak, while level I evidence is derived either from at least one prospective randomised controlled trial specifically designed to test the marker or from a meta-analysis and/or overview of level II or III studies and is considered definitive (4). In addition, a consortium of the National Cancer Institute-European Organisation for Research and Treatment of Cancer (NCI-EORTC) reported in several journals a guideline for reporting tumour marker prognostic studies (REMARK) (5).

In this review, the progress in the development of biomarkers in solid tumours will be addressed such as, involvement of cellular pathways in tumour biology instead of single genes and interference in disease outcome as a result of anticancer therapies. Examples of both well-known biomarkers and potential new discoveries (summarised in Table 1) will form the basis for a discussion below on the present knowledge and new avenues for the development of prognostic and predictive biomarkers.

ER/PR in breast cancer

ER and/or PR expression is an independent prognostic factor in breast cancer. Patients with ER and/or PR positive tumours have a better survival than hormone receptor negative tumours, with a 5-year overall survival (all stages) of 83% in the ER+/PR+ group versus 69% in the double negatives (LOE III) (6). High cellular expression of ER and PR predicts benefit from endocrine therapy in the adjuvant and metastatic setting (LOE I) (7). Tumour hormone receptor status is, therefore, routinely assessed in breast cancer. It now also becomes clear that hormone receptor status in a patient can change during the course of the disease and may differ across lesions. For example, the ER status of metastatic disease is different from the primary tumour in about 20% of cases (8,9). In addition, PR expression is lost in 40% of previous positive tumours when they metastasise. Therefore, recently revised guidelines of the American Society of Clinical Oncology recommend measurement of both ER and PR in metastatic lesions if these results might influence treatment planning (10). A search for non-invasive techniques to predict response to treatment is ongoing. Studies with positron emission tomography (PET) for whole body ER imaging with [^{18}F]fluoroestradiol (FES) suggest feasibility of such an approach (11).

HER2/neu in breast cancer

Another relevant biomarker in breast cancer patients is HER2/neu. The *HER2/neu* gene amplification leads to overexpression of its receptor on the cell membrane. This results in increased proliferation and angiogenesis, and inhibition of apoptosis. HER2/neu positive tumours are more aggressive and have, therefore, a worse prognosis compared to negative tumours. In this respect, HER2/neu in node positive breast cancer is of prognostic value (LOE II). For the node negative, HER2/neu positive group of patients this is less clear (12-17). HER2/neu is the target for the monoclonal antibody trastuzumab and the EGFR1 and HER2 dual tyrosine kinase inhibitor (TKI) lapatinib. Patients with HER2/neu overexpressing tumours benefit from treatment with trastuzumab in the metastatic as well as in the adjuvant setting (LOE II) (18-20). Interestingly, HER2/neu positive patients receiving adjuvant chemotherapy plus

trastuzumab showed the same recurrence-free survival as HER2/neu negative patients treated with chemotherapy alone (21). Thus, the prognostic value of HER2/neu overexpression is neutralised in this study by targeting the prognostic biomarker.

Like ER expression, HER2/neu expression can change over time and can vary between lesions within a patient. Several reports suggest a conversion from negative into positive HER2/neu status when the disease did recur, although depending on technique, for a varying percentage (22-25). Instead of serial biopsies, SPECT or PET whole body radiolabelled trastuzumab scintigraphy would be more attractive. This approach was capable to detect HER2/neu expression in tumour lesions in the patients (26,27).

Both primary and acquired resistance to trastuzumab occurs. In addition to absence of the receptor there are a number of other factors that can potentially explain resistance. For example, the presence of multiple truncated HER2/neu receptors at the tumour cell surface might play a role. The truncated p95HER2/neu receptor lacks the extracellular binding domain for trastuzumab, but has tyrosine kinase activity. Therefore, trastuzumab resistant tumours that express p95HER2/neu might benefit from treatment with lapatinib (28).

Prognostic biomarkers for the relapse of breast cancer

Decision making about adjuvant systemic treatment for breast cancer is based on nodal status, tumour grade, tumour size, tumour hormone receptor and HER2/neu status, age and co-morbidity. Prognostic biomarkers that could provide better information on risk of relapse could spare many patients chemotherapy toxicity without compromising survival. Among several initiatives Buyse and colleagues validated a 70-gene signature for node negative breast cancer patients that has independent prognostic value additive to clinicopathologic parameters (29). This approach is now tested in a prospective European study (MINDACT).

Table 1. Biomarkers of interest: overview of prognostic and predictive value.

Biomarker	**Tumour type**	**Prognostic value**		**Predictive value**		
			LOE		***LOE***	***Therapy***
ER/PR	BRCA	Yes	III	Yes	I	Endocrine therapy
HER2/neu	BRCA	Yes	II	Yes	II	Trastuzumab
c-KIT	GIST	Yes, subgroup[1]	II	Yes, subgroup[2]	II	Imatinib
EGFR1	NSCLC	No	III	Yes, subgroup[3]	II	Gefitinib, erlotinib
	CRC	No	III	Yes	IV	Cetuximab, panitumumab
Mutated K-ras	NSCLC	Yes	II	Yes	III	Gefitinib, erlotinib
	CRC	No	III	Yes	IV	Cetuximab, panitumumab
TRAIL receptors	CRC	Yes	II	NK	-	RhTRAIL; TRAIL receptor antibodies
VEGF	RCC	Yes	II	No	II	Angiogenesis inhibitors

LOE: level of evidence; ER: oestrogen receptor; PR: progesterone receptor; BRCA: breast cancer; GIST: gastrointestinal stromal tumours; EGFR1: epidermal growth factor receptor 1; NSCLC: non-small cell lung cancer; CRC: colorectal cancer; TRAIL: tumour necrosis factor (TNF)-related apoptosis-inducing ligand; NK: not known; VEGF: vascular endothelial growth factor; RCC renal cell carcinoma.

1. c-KIT exon 11 mutation.

2. c-KIT exon 9 mutation.

3. EGFR1 exon 18, 19 or 21 mutation.

c-KIT in GIST

Several features are evaluated over the last few years to determine malignant behaviour of GISTs. Of known relevance are tumour size and mitotic index, which are used to classify the biologic behaviour of GIST (30). The majority of GISTs are characterised by mutations in either the proto-oncogene *c-KIT* or the *platelet-derived growth factor receptor alpha* (*PDGFRα*). Interestingly, patients with mutation in the *c-KIT*-gene in exon 11 have a better prognosis as compared to those who lack a mutation or have another mutation (LOE II) (31-33). With the introduction of imatinib and sunitinib the outcome of GIST patients improved dramatically (34,35). Imatinib and sunitinib are small molecule TKIs, which block signalling via c-KIT and PDGFRα. In 50-55% of the patients with advanced disease imatinib results in a durable objective response, while another 25-30% have stable disease according to RECIST (36). During the course of treatment, however, most patients develop resistance to imatinib. A subgroup of these patients with progressive disease within a few months of imatinib treatment was characterised by bearing exon 9 activating mutations in *c-KIT*. In the two studies comparing an imatinib dose of 400 and 800 mg daily, the only difference was a better progression-free survival at the highest dose in patients with a tumour harbouring an exon 9 mutation (LOE II) (32). Exon 9 mutational status is, therefore, a negative predictive factor for response to imatinib and a positive predictive factor for benefit of 800 mg imatinib (37). Progression after an initial response or stable disease for at least 3 months is caused by secondary *c-KIT* mutations in exon 13, 14, 17 or 18 in 50-70% of these patients (38). Secondary mutations can differ across lesions in an individual patient. Several studies explored the value of different mutations in *c-KIT* and *PDGFRα* in the light of predicting response to TKIs (39).

EGFR1 and K-ras in NSCLC and colorectal cancer (CRC)

In NSCLC and CRC, biomarkers of interest are EGFR1 and the *K-ras* oncogene. EGFR1 is overexpressed in multiple cancer types and is one of the targets in the treatment of NSCLC and metastatic CRC. The EGFR pathway plays a role in several cellular functions,

including regulation of cell proliferation, migration and differentiation (Figure 1). The prognostic value of EGFR1 protein expression is extensively studied in NSCLC and CRC patients but no definitive association between EGFR1 expression and prognosis was found (40,41).

The *K-ras* oncogene controls cell growth via regulation of signal transduction pathways. *K-ras* mutation results in malignant transformation. In a meta-analysis including 28 studies assessing the correlation between *K-ras* mutation and survival in NSCLC patients, *K-ras* mutation appeared to be a biomarker of poor prognosis (42). A multivariate analysis including 3439 CRC patients failed to prove an association between mutant *K-ras* and disease outcome (43).

In recent years, two small-molecule EGFR1 TKIs (gefitinib and erlotinib) and two anti-EGFR1 monoclonal antibodies (cetuximab and panitumumab) were introduced in the clinic. Four phase III trials in previously untreated patients with advanced NSCLC combining two different chemotherapy regimens with and without gefitinib or erlotinib demonstrated no survival benefit. Subgroup analysis, however, identified four characteristics associated with benefit for the patient namely adenocarcinoma, female sex, Asian ethnicity and non-smoking (44). Over the last years, it became clear that a subgroup of NSCLC patients, especially consisting of non-smokers, have mutations in the tyrosine kinase domain (exons 18, 19, and 21) of *EGFR1*. These mutations are predictive for response to either gefitinib or erlotinib (LOE II) (45-48). In contrast to *EGFR1* mutations being a predictive biomarker for a beneficial effect, mutations in *K-ras* are most commonly observed in heavy smokers predicting for treatment failure on EGFR1 TKIs (49,50).

In metastatic CRC, a subgroup of patients benefits of EGFR1 directed antibody treatment. However, *EGFR1* mutations are rare in CRC patients and do not predict benefit from anti-EGFR1 therapy. In contrast, *EGFR1* gene amplification appears to be a predictive factor for response to anti-EGFR1 antibody treatment in CRC, although the studied series are small and retrospective (LOE IV) (40). In CRC, there is also increasing evidence that mutations in *K-ras* are predictive of non-response to cetuximab and/or panitumumab (40,51,52).

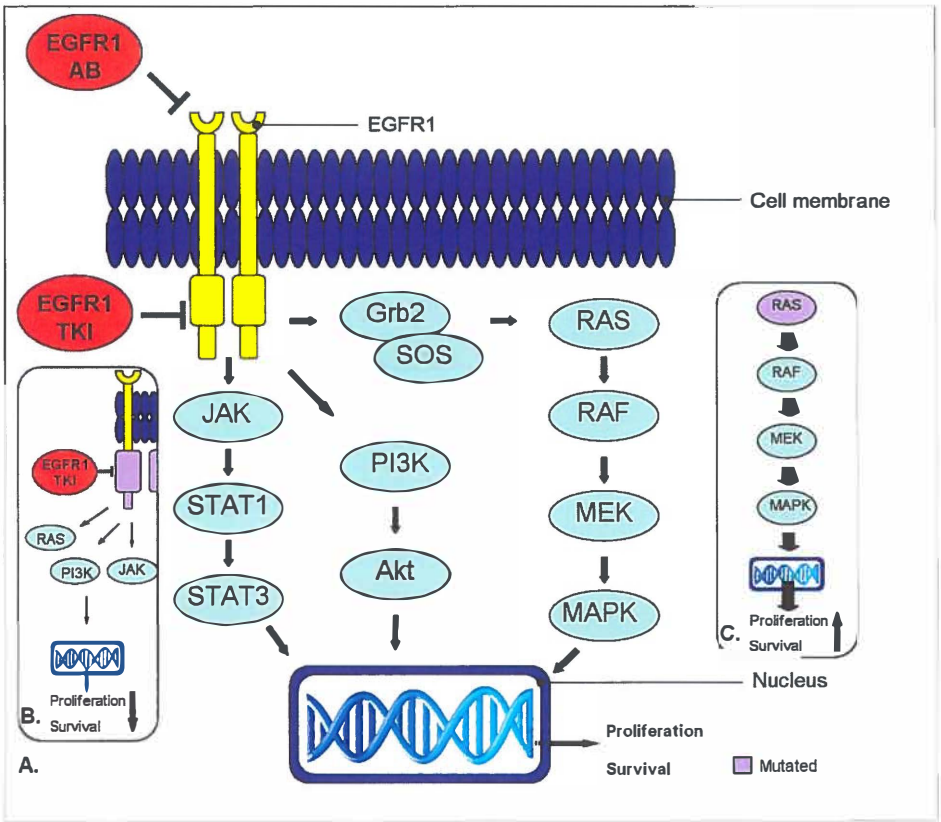


Figure 1.

A. EGFR1 pathway. EGFR1 forms homodimers after binding by its growth factor ligands. This results in stimulation of intrinsic tyrosine kinase (TK) activity. Subsequently, several downstream signal transduction pathways are initiated resulting in the cell proliferation and the cell survival. EGFR1 antibodies and TK inhibitors can block signalling by binding to the extracellular domain and the intracellular TK domain, respectively. AB: antibody.

B. Mutations in the tyrosine kinase domain (exons 18, 19 and 21) of EGFR1 result in a better response to EGFR1 TKIs.

C. Mutated RAS results in continuous signalling, independent of EGFR1 and EGFR1 targeting agents.

TRAIL receptors

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL or Apo2L) induces apoptosis in a wide variety of tumour cell lines without causing toxicity to

normal cells and is, therefore, a potential attractive agent. TRAIL binds the death receptors TRAIL-R1 (DR4) and TRAIl-R2 (DR5) and initiates the apoptotic pathway. DR4 and DR5 are expressed on most tumour cells. In contrast to, e.g., HER2 and EGFR1, this receptor has to be activated and not inhibited in order for cells to go into apoptosis.

Several studies addressed the prognostic role of DR expression in malignancies. In 376 stage III CRC patients receiving adjuvant chemotherapy as part of a randomised study, high DR4 expression was associated with a worse disease-free and overall survival (LOE II) (53). RhTRAIL and several agonistic antibodies targeting the TRAIL receptors are currently evaluated in the clinic (54). So far, very little is known about possible predictive factors, and only pre-clinical data are available. DR4 and/or DR5 have to be available at the tumour cell surface to initiate the apoptotic pathway, but just their presence is not sufficient to predict response to TRAIL receptor targeting agents. Several downstream factors in the TRAIL signalling pathway, for example, defects in caspase 8 or loss of function of the pro-apoptotic proteins BAK or BAX determine apoptotic response to TRAIL (55). In addition, mutations in *DR5* are responsible for inhibition of apoptosis by blocking the signal after TRAIL binding (56). An interesting biomarker is the *O*-glycosylation status of DRs. O-linked glycans regulate biochemical and functional properties of cell surface proteins, including apoptosis. *O*-Glycosyltransferase mRNA levels correlated with rhTRAIL sensitivity in several cancer cell lines and DR *O*-glycosylation resulted in the activation of caspase 8 via TRAIL induced clustering of DR4 and DR5 (57). In an attempt to predict which patients might benefit TRAIL receptors targeting therapy, a SPECT imaging study with the radioactively labelled DR4 agonistic antibody mapatumumab is initiated.

VEGF and renal cell carcinoma (RCC)

Even very small tumours require angiogenesis to provide nutrients and oxygen for survival. There is a close interaction between tumour cells that produce pro-angiogenic growth factors, like vascular endothelial growth factor (VEGF) and PDGF, and endothelial cells expressing growth factor receptors. The stimulation of endothelial cells results in proliferation and migration and eventually in formation of new vessels.

Clear cell RCC provides a unique model for studying angiogenesis because of frequent somatic inactivation of the *Von Hippel Lindau* (*VHL*) gene. The *VHL* gene plays a key role in regulation of the oxygen-sensing pathway by targeting the hypoxia-inducible factor (HIF) for degradation in the proteasome. Impaired VHL function, therefore, results in high expression of pro-angiogenic growth factors. Targeting the VEGF pathway with TKI sunitinib and sorafenib, the mTOR inhibitor temsirolimus and with the VEGF targeting monoclonal antibody bevacizumab prolongs progression-free survival in metastatic clear cell RCC (58-62).

Simple clinical parameters like performance score and number of metastatic sites are known powerful prognostic factors in cancer. Motzer and colleagues developed a scoring system for metastatic RCC patients consisting of five clinical parameters: performance score, time between diagnosis and metastasis, haemoglobin, serum calcium and lactate dehydrogenase (LDH) (63). With this system, developed retrospectively in patients receiving interferon, patients can be classified as having good, intermediate or poor prognosis (LOE II) (4). This classification has been used to design and stratify medical intervention studies and as a consequence is now widely used to guide therapy.

Several other parameters like C-reactive protein, platelet count and HIF expression also have prognostic value in RCC but do not have a role in clinical decision making.

A high baseline serum VEGF level is associated with shorter progression free and overall survival in 2 prospective studies (4,64). In a phase III study of sorafenib versus placebo in advanced RCC, baseline VEGF level is an independent prognostic factor for overall survival (LOE II). Baseline serum VEGF in 2 cytokine studies in RCC patients also found VEGF to be an independent prognostic factor for survival (LOE III). No predictive biomarkers have been found so far which predict patients' benefit from angiogenesis inhibitors (65,66). VEGF mRNA and protein levels in serum, plasma and tumour have been investigated extensively with disappointing results. Even in RCC, the role model for angiogenesis, a high serum VEGF level or a change after starting therapy does not predict response to anti-angiogenic treatment. In the sorafenib study, both patients with high and patients with low baseline serum VEGF benefited from sorafenib (64).

There is a growing list of candidate markers from pre-clinical studies, and multiple clinical trials are underway to assess predictive biomarkers for angiogenesis inhibition. Single molecular markers may not be able to predict the benefit because of the complexity of signalling routes and because of the cross talk between different signalling pathways.

Functional imaging with MRI, CT and PET scans for the assessment of tumour vascularity and metabolic activity is under investigation for its ability to predict response to angiogenesis inhibitors earlier. In vivo imaging of VEGF by radiolabelled bevacizumab has been successful in a human ovarian tumour xenograft and is an interesting concept for early response prediction (67).

Drug induced toxicity as a predictive biomarker

Interestingly a number of studies showed that the effect of a drug on normal tissues can be used as biomarker. In both a phase II and a phase III study evaluating the antitumour activity of cetuximab in metastatic CRC, skin rash was strongly related to response and survival (68,69). Similar results were found for erlotinib in NSCLC (70). Toxicity might thus be used to titrate drugs to effective doses as is done in the EVEREST study in CRC patients. Patients with no or mild skin toxicity after 22 days of treatment with cetuximab and irinotecan were randomised between standard and escalating doses of cetuximab until the development of grade 3 toxicity. Preliminary data show that dose escalation improves tumour response to a rate comparable to the group with initial moderate to severe skin toxicity at the standard dose (71).

In a small series of 40 metastatic RCC patients treated with sunitinib, grade 3 hypertension was associated with a higher objective response (72).

Discussion

A confusing mix-up exists of the terms prognostic and predictive biomarkers. This is partially due to the fact that predictive and prognostic biomarkers are frequently exchanged. In addition, during therapy or as a result of therapy initial factors can vary

in their presence and actual levels, e.g. a strong prognostic factor can be neutralised as a consequence of treatment (HER2/neu).

Despite a growing number of publications about biomarkers that give information on disease outcome, the best prognostic factors are still simple clinical parameters like performance status, number of metastatic sites, tumour grade and LDH level. Prognostic biomarkers might especially be useful for hypothesis testing for their relevance as predictive markers, as targets for therapy and for the selection of patients for adjuvant treatment.

What we need is predictive biomarkers that can guide patient tailored therapy as with our increasing knowledge of biologic behaviour of malignancies it becomes more and more evident that great heterogeneity among tumours exists. Together with the development of new anticancer biologicals an explosive search for effective predictive biomarkers has been initiated. Most studies only contribute low levels of evidence due to retrospective data and small sample size. In addition, many reports lack sufficient information to be compared to other studies, and it is therefore difficult to form an opinion about usefulness of such markers in daily practice. A predictive factor is used upfront to predict response to therapy or is monitored during treatment to define the effectiveness of this treatment. When a biomarker is used repeatedly to evaluate response, it is important that it can be measured non-invasively and gives information on all tumour lesions. In this perspective, and also for the evaluation of biomarker conversion during the course of the disease, there might be a role for imaging techniques to quantify levels of biomarkers over time for certain therapies.

Different tumour types can be treated by blocking the same pathway. Predictive biomarkers may be shared between tumour types, like the negative predictive value of K-ras mutations in CRC and NSCLC for benefit from EGFR1 inhibition. However, EGFR1 mutations do predict benefit from EGFR1 directed therapy in NSCLC but not in CRC.

Response to c-KIT and EGFR1 targeting agents in GIST and NSCLC cannot be predicted by the expression of their respective receptors, only by analysing specific mutations in the genes encoding for these receptors. This research finally might pay off as it will

allow specific selection of patients that will benefit from the TKIs at a certain dose-level.

For EGFR targeting agents and angiogenesis inhibitors we presented studies that indicate that apart from the tumour also drug effects on normal tissues can be used as a predictive factor (68-70,72). Preliminary data in CRC patients in which the dose of cetuximab was titrated to skin rash suggests improvement of tumour response rate (71).

These findings show that toxicity caused by the drug can be an early predictive factor for response. This is of great interest, because such clinical phenomena are much cheaper, always available and may be easier to exploit than the previously discussed genes or their products.

Biomarkers are in general based on single markers. However, given the fact that tumour biology is often dictated by several essential cellular alterations it may be idle to think that single factors will be enough as predictive or prognostic factors in oncology. Solutions are now sought by analysing multiple factors with multiple reverse transcriptase-polymerase chain reaction (RT-PCRs), RNA microarrays and tissue microarrays. Significant contributions have already been made in the area of breast cancer research. Paik and colleagues tested whether the results of a RT-PCR assay of 21 prospectively selected genes in paraffin-embedded tumour tissue would correlate with the likelihood of distant recurrence in patients with node-negative, tamoxifen-treated breast cancer who were enrolled in the National Surgical Adjuvant Breast and Bowel trial B-14. RT-PCR of the selected genes was significant in predicting recurrence and overall survival (73). Other microarray studies in breast cancer identified independent sets of genes that might have prognostic value (29,74-76).

Until recently, analysis was directed at identifying major differences in the expression of separate genes. Currently, there is increasing interest in minor changes in related genes that are involved in particular signalling pathways. Elucidation of pathways that are dysregulated in a specific tumour may lead to rational treatment selection (77).

In conclusion, prognostic biomarkers for relapse after local treatment are needed for better patient selection for adjuvant treatment strategies. Discovery of prognostic factors in the metastatic setting may identify new therapeutic targets and new predictive factors. The need for more upfront predictive biomarkers to select patients for tailored therapy is clear. Clinical observations during treatment can contribute to the identification of upfront predictive biomarkers, like EGFR1-mutations in non-smoking NSCLC patients. In addition, early predictive markers might be useful in dose selection and early response measurement, because classical response measurement by RECIST criteria underestimates the clinical benefit of the new biological agents. Progress is made, but there is still an urgent need for prospective data to validate all the small, hypotheses generating studies and it is therefore of great importance that biomarker analyses are incorporated in randomised clinical trials as a separate objective.

Conflict of interest statement

None declared.

References

1. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 2001;69:89-95.
2. NIH consensus conference. Treatment of early-stage breast cancer. JAMA 1991;265:391-5.
3. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57-70.
4. Hayes DF, Bast RC, Desch CE, et al. Tumor marker utility grading system: a framework to evaluate clinical utility of tumor markers. J Natl Cancer Inst 1996; 88:1456-66.
5. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005;97:1180-4.
6. Grann VR, Troxel AB, Zojwalla NJ, Jacobson JS, Hershman D, Neugut AI. Hormone receptor status and survival in a population-based cohort of patients with breast carcinoma. Cancer 2005;103:2241-51.
7. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687-717.
8. Franco A, Col N, Chlebowski RT. Discordance in estrogen (ER) and progestin receptor (PR) status between primary metastatic breast cancer: a meta-analysis. J Clin Oncol 2004;22:14s [abstract 539].
9. Kuukasjarvi T, Kononen J, Helin H, Holli K, Isola J. Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. J Clin Oncol 1996;14:2584-9.
10. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol 2007;25:5287-312.
11. Linden HM, Stekhova SA, Link JM, et al. Quantitative fluoroestradiol positron emission tomography imaging predicts response to endocrine treatment in breast cancer. J Clin Oncol 2006;24:2793-9.
12. Gusterson BA, Gelber RD, Goldhirsch A, et al. Prognostic importance of c-erbB-2 expression in breast cancer. International (Ludwig) Breast Cancer Study Group. J Clin Oncol 1992;10:1049-56.
13. Tandon AK, Clark GM, Chamness GC, Ullrich A, McGuire WL. HER-2/neu oncogene protein and prognosis in breast cancer. J Clin Oncol 1989;7:1120-8.
14. Kroger N, Milde-Langosch K, Riethdorf S, et al. Prognostic and predictive effects of immunohistochemical factors in high-risk primary breast cancer patients. Clin Cancer Res 2006;12:159-68.
15. Clark GM, McGuire WL. Follow-up study of HER-2/neu amplification in primary breast cancer. Cancer Res 1991;51:944-8.
16. Paterson MC, Dietrich KD, Danyluk J, et al. Correlation between c-erbB-2 amplification and risk of recurrent disease in node-negative breast cancer. Cancer Res 1991;51:556-67.
17. Winstanley J, Cooke T, Murray GD, et al. The long term prognostic significance of c-erbB-2 in primary breast cancer. Br J Cancer 1991;63:447-50.
18. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. New Engl J Med 2005;353:1673-84.
19. Smith I, Procter M, Gelber RD, et al. 2-Year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. Lancet 2007;369:29-36.
20. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol 2002;20:719-26.
21. Joensuu H, Kellokumpu-Lehtinen PL, Bono P, et al. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. New Engl J Med 2006;354:809-20.
22. Gancberg D, Di LA, Cardoso F, et al. Comparison of HER-2 status between primary breast cancer and corresponding distant metastatic sites. Ann Oncol 2002;13:1036-43.
23. Gong Y, Booser DJ, Sneige N. Comparison of HER-2 status determined by fluorescence in situ hybridization in primary and metastatic breast carcinoma. Cancer 2005;103:1763-9.
24. Meng S, Tripathy D, Shete S, et al. HER-2 gene amplification can be acquired as breast cancer progresses. Proc Natl Acad Sci USA 2004;101:9393-8.

25. Zidan J, Dashkovsky I, Stayerman C, Basher W, Cozacov C, Hadary A. Comparison of HER-2 overexpression in primary breast cancer and metastatic sites and its effect on biological targeting therapy of metastatic disease. Br J Cancer 2005;93:552-6.
26. Harris LN, Liotcheva V, Broadwater G, et al. Comparison of methods of measuring HER-2 in metastatic breast cancer patients treated with high-dose chemotherapy. J Clin Oncol 2001;19:1698-706.
27. Perik PJ, Lub-De Hooge MN, Gietema JA, et al. Indium-111-labeled trastuzumab scintigraphy in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer. J Clin Oncol 2006;24:2276-82.
28. Scaltriti M, Rojo F, Ocana A, et al. Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. J Natl Cancer Inst 2007;99:628-38.
29. Buyse M, Loi S, van't Veer L, et al. Validation and clinical utility of a 70-gene prognostic signature for women with node-negative breast cancer. J Natl Cancer Inst 2006;98:1183-92.
30. Fletcher CD, Berman JJ, Corless C, et al. Diagnosis of gastrointestinal stromal tumors: a consensus approach. Int J Surg Pathol 2002;10:81-9.
31. Singer S, Rubin BP, Lux ML, et al. Prognostic value of KIT mutation type, mitotic activity, and histologic subtype in gastrointestinal stromal tumors. J Clin Oncol 2002;20:3898-905.
32. Van Glabbeke MM, Owzar K, Rankin C, Simes J, GIST Meta-analysis Group (MetaGIST). Comparison of two doses of imatinib for the treatment of unresectable or metastatic gastrointestinal stromal tumors (GIST): a meta-analyis based on 1,640 patients (pts). J Clin Oncol 2007;25:18s [abstract 10004].
33. Kim TW, Lee H, Kang YK, et al. Prognostic significance of c-kit mutation in localized gastrointestinal stromal tumors. Clin Cancer Res 2004;10:3076-81.
34. Demetri GD, von Mehren M, Blanke CD, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. New Engl J Med 2002;347:472-80.
35. Demetri GD, van Oosterom AT, Garrett CR, et al. Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. Lancet 2006;368:1329-38.
36. Verweij J, Casali PG, Zalcberg J, et al. Progression-free survival in gastrointestinal stromal tumours with high-dose imatinib: randomised trial. Lancet 2004;364:1127-34.
37. Debiec-Rychter M, Sciot R, Le Cesne A, et al. KIT mutations and dose selection for imatinib in patients with advanced gastrointestinal stromal tumours. Eur J Cancer 2006;42:1093-103.
38. Sleijfer S, Wiemer E, Seynaeve C, Verweij J. Improved insight into resistance mechanisms to imatinib in gastrointestinal stromal tumors: a basis for novel approaches and individualization of treatment. Oncologist 2007;12:719-26.
39. Wardelmann E, Buttner R, Merkelbach-Bruse S, Schildhaus HU. Mutation analysis of gastrointestinal stromal tumors: increasing significance for risk assessment and effective targeted therapy. Virchows Arch 2007;451:743-9.
40. Ng K, Zhu AX. Targeting the epidermal growth factor receptor in metastatic colorectal cancer. Crit Rev Oncol Hematol 2008;65:8-20.
41. Scagliotti GV, Selvaggi G, Novello S, Hirsch FR. The biology of epidermal growth factor receptor in lung cancer. Clin Cancer Res 2004;10:4227s-32s.
42. Mascaux C, Iannino N, Martin B, et al. The role of RAS oncogene in survival of patients with lung cancer: a systematic review of the literature with meta-analysis. Br J Cancer 2005;92:131-9.
43. Andreyev HJ, Norman AR, Cunningham D, et al. Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. Br J Cancer 2001;85:692-6.
44. Sequist LV, Bell DW, Lynch TJ, Haber DA. Molecular predictors of response to epidermal growth factor receptor antagonists in non-small-cell lung cancer. J Clin Oncol 2007;25:587-95.
45. Herbst RS, Prager D, Hermann R, et al. TRIBUTE: a phase III trial of erlotinib hydrochloride (OSI-774) combined with carboplatin and paclitaxel chemotherapy in advanced non-small-cell lung cancer. J Clin Oncol 2005;23:5892-9.
46. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. New Engl J Med 2004;350:2129-39.

47. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA 2004;101:13306-11.
48. Pham D, Kris MG, Riely GJ, et al. Use of cigarette-smoking history to estimate the likelihood of mutations in epidermal growth factor receptor gene exons 19 and 21 in lung adenocarcinomas. J Clin Oncol 2006;24:1700-4.
49. Eberhard DA, Johnson BE, Amler LC, et al. Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. J Clin Oncol 2005;23:5900-9.
50. Pao W, Miller VA. Epidermal growth factor receptor mutations, small-molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. J Clin Oncol 2005;23:2556-68.
51. Amado RG, Wolf M, Freeman D, et al. Panitumumab (pmab) efficacy and patient-reported outcomes (PRO) in metastatic colorectal cancer (mCRC) patients (pts) with wild-type (WT) KRAS tumor status. Gastrointestinal Cancers Symposium; 2008 [abstract 278].
52. Khambata-Ford S, Garrett CR, Meropol NJ, et al. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. J Clin Oncol 2007;25:3230-7.
53. Van Geelen CM, Westra JL, de Vries EG, et al. Prognostic significance of tumor necrosis factor-related apoptosis-inducing ligand and its receptors in adjuvantly treated stage III colon cancer patients. J Clin Oncol 2006;24:4998-5004.
54. Duiker EW, Mom CH, de Jong S, et al. The clinical trail of TRAIL. Eur J Cancer 2006;42:2233-40.
55. Carlo-Stella C, Lavazza C, Locatelli A, Vigano L, Gianni AM, Gianni L. Targeting TRAIL agonistic receptors for cancer therapy. Clin Cancer Res 2007;13:2313-7.
56. Bin L, Thorburn J, Thomas LR, Clark PE, Humphreys R, Thorburn A. Tumor-derived mutations in the TRAIL receptor DR5 inhibit TRAIL signaling through the DR4 receptor by competing for ligand binding. J Biol Chem 2007;282:28189-94.
57. Wagner KW, Punnoose EA, Januario T, et al. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nat Med 2007;13:1070-7.
58. Escudier B, Eisen T, Stadler WM, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. New Engl J Med 2007;356:125-34.
59. Escudier B, Koralewski P, Pluzanska A, et al. A randomized, controlled, double-blind phase III (AVOREN) study of bevacizumab/interferon-alpha2a vs placebo/interferon-alpha2a as first-line therapy in metastatic renal cell carcinoma. J Clin Oncol 2007;25:18s [abstract 3].
60. Hudes G, Carducci M, Tomczak P, et al. Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. New Engl J Med 2007;356:2271-81.
61. Motzer RJ, Hutson TE, Tomczak P, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. New Engl J Med 2007;356:115-24.
62. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. New Engl J Med 2003;349:427-34.
63. Motzer RJ, Bacik J, Murphy BA, Russo P, Mazumdar M. Interferon-alfa as a comparative treatment for clinical trials of new therapies against advanced renal cell carcinoma. J Clin Oncol 2002;20:289-96.
64. Bukowski R, Eisen T, Szczylik C, et al. Final results of the randomized phase III trial of sorafenib in advanced renal cell carcinoma. Survival and biomarker analysis. J Clin Oncol 2007;25:18s [abstract 5023].
65. Jubb AM, Oates AJ, Holden S, Koeppen H. Predicting benefit from anti-angiogenic agents in malignancy. Nat Rev Cancer 2006;6:626-35.
66. Longo R, Gasparini G. Challenges for patient selection with VEGF inhibitors. Cancer Chemother Pharmacol 2007;60:151-70.
67. Nagengast WB, de Vries EG, Hospers GA, et al. In vivo VEGF imaging with radiolabeled bevacizumab in a human ovarian tumor xenograft. J Nucl Med 2007;48:1313-9.

68. Lenz HJ, Van Cutsem E, Khambata-Ford S, et al. Multicenter phase II and translational study of cetuximab in metastatic colorectal carcinoma refractory to irinotecan, oxaliplatin, and fluoropyrimidines. J Clin Oncol 2006;24:4914-21.
69. Van Cutsem E, Nowacki M, Lang I, et al. Randomized phase III study of irinotecan and 5-FU/FA with or without cetuximab in the first-line treatment of patients with metastatic colorectal cancer (mCRC): The CRYSTAL trial. J Clin Oncol 2007;25:18s [abstract 4000].
70. Perez-Soler R, Chachoua A, Hammond LA, et al. Determinants of tumor response and survival with erlotinib in patients with non-small-cell lung cancer. J Clin Oncol 2004;22:3238-47.
71. Van Cutsem E, Humblet Y, Gelderblom H, et al. Cetuximab dose-escalation study in patients with metastatic colorectal cancer (mCRC) with no or slight skin reactions on cetuximab standard dose treatment (EVEREST): Pharmacokinetic and efficacy data of a randomized study. Gastrointestinal cancers symposium; 2007. [abstract 237]
72. Rixe O, Billemont B, Izzedine H. Hypertension as a predictive factor of sunitinib activity. Ann Oncol 2007;18:1117.
73. Paik S, Shak S, Tang G, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. New Engl J Med 2004;351:2817-26.
74. Foekens JA, Atkins D, Zhang Y, et al. Multicenter validation of a gene expression-based prognostic signature in lymph node-negative primary breast cancer. J Clin Oncol 2006;24:1665-71.
75. Goetz MP, Suman VJ, Ingle JN, et al. A two-gene expression ratio of homeobox 13 and interleukin-17B receptor for prediction of recurrence and survival in women receiving adjuvant tamoxifen. Clin Cancer Res 2006;12:2080-7.
76. Jansen MP, Sieuwerts AM, Look MP, et al. HOXB13-to-IL17BR expression ratio is related with tumor aggressiveness and response to tamoxifen of recurrent breast cancer: a retrospective study. J Clin Oncol 2007;25:662-8.
77. Bild AH, Potti A, Nevins JR. Linking oncogenic pathways with therapeutic opportunities. Nat Rev Cancer 2006;9:735-41.

CHAPTER 6

Sulindac inhibits ß-catenin expression in normal-appearing colon of Hereditary Nonpolyposis Colorectal Cancer and Familial Adenomatous Polyposis patients

JJ Koornstra,[1] FEM Rijcken,[1] CNAM Oldenhuis,[1] N Zwart,[2] T van der Sluis,[3] H Hollema,[3] EGE de Vries,[2] JJ Keller,[4] JA Offerhaus,[4] FM Giardiello,[5] JH Kleibeuker[1].

Departments of [1]Gastroenterology, [2]Medical Oncology, and [3]Pathology, University Medical Center Groningen, Groningen, the Netherlands; [4]Department of Pathology, Academic Medical Center, Amsterdam, the Netherlands; and [5]Department of Medicine, Johns Hopkins Medical Institutions, Baltimore, Maryland.

Cancer Epidemiology, Biomarkers & Prevention. 2005;14:1608-12.

Abstract

Sulindac reduces colorectal cancer risk in genetically susceptible humans and animals. The molecular mechanisms underlying these effects are incompletely understood. Many studies suggest an important role for induction of apoptosis involving the mitochondrial pathway and the death receptor pathway. Alternatively, mechanisms involving the APC-β-catenin-Wnt pathway have been suggested, possibly mediated by p21. We determined the effects of sulindac on apoptosis and expression of death receptor (DR)-4 and DR5, β-catenin, and p21 in normal-appearing colorectal epithelium. Biopsies were obtained before and after sulindac treatment during two chemoprevention studies. Patients (n = 18) with hereditary nonpolyposis colorectal cancer (HNPCC) received 150 mg sulindac bd for 4 weeks in a placebo-controlled crossover design. Patients (n = 6) with familial adenomatous polyposis (FAP) received 150 mg sulindac bd for 6 months. Apoptosis was assessed by M30 staining and expression patterns of DR4, DR5, β-catenin, and p21 were studied immunohistochemically. In HNPCC patients, apoptotic indices were similar following placebo and sulindac. Also in FAP patients, apoptotic indices were not different after sulindac compared with pretreatment values. Expression of DR4 and DR5 was observed in all samples with no consistent differences between placebo/baseline and sulindac. Intensity of membranous β-catenin staining was lower in HNPCC samples following sulindac compared with placebo ($P < 0.001$). Similar results were obtained in FAP samples ($P < 0.01$). p21 expressions before and after sulindac treatment were similar in both patient groups. In conclusion, sulindac inhibits β-catenin expression in normal colorectal epithelium from HNPCC and FAP patients without affecting apoptotic indices and DR4, DR5, and p21 expression.

Introduction

Colorectal cancer is the second leading cause of cancer death in the Western world. Familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) are well-defined conditions predisposing to colorectal cancer (1). Numerous studies have established the chemopreventive effects of nonsteroidal anti-inflammatory drugs (NSAIDs) as sulindac (2-4) and celecoxib (5) in patients with FAP, whereas studies in HNPCC patients are ongoing (6).

The precise mechanisms by which NSAIDs mediate their effects are incompletely understood, but likely involve induction of apoptosis (7-9). Apoptosis is controlled via two major pathways, one originating at the cell membrane, the extrinsic pathway, and one involving the mitochondria, the intrinsic pathway (10). Apoptotic pathways originating at the cell membrane involve death receptors like Fas, tumor necrosis factor receptor 1, death receptor (DR)-3, DR4, and DR5, which are activated on binding to their respective ligands (10). Recent reports reveal that the NSAID sulindac mediates apoptosis through the mitochondrial pathway in colon cancer cells, involving caspase 9 and BAX (11). Sulindac-induced apoptosis has also been shown to involve DR5, as sulindac induced up-regulation of DR5 mRNA and protein levels, but not of DR4, in vitro (12,13).

The initial event in the neoplastic transformation of normal colon epithelium is assumed to be the activation of the Wnt signaling pathway, caused by mutations in the *APC* or the *β-catenin* gene (14). This leads to cytoplasmic and subsequent nuclear accumulation of β-catenin. In the nucleus, β-catenin binds and activates the transcription factor T-cell factor 4 (TCF4). Finally, activated TCF4 activates a genetic program presumed to be responsible for early adenoma formation (14). Several in vitro studies suggest that sulindac mediates its antineoplastic effect by inhibition of the Wnt pathway (15-17). This is supported by studies in APC^{min} mice (18) and in adenomas of FAP patients (15). Recent reports reveal that sulindac affects Wnt signaling by modifying expression of p21, a cyclin-dependent kinase inhibitor (19).

To provide further insight into the mechanisms involved in the chemopreventive action of sulindac, we investigated the effects on apoptosis and expression of DR4, DR5, β-catenin, and p21 in normal epithelium of FAP and HNPCC patients.

Materials and methods

Patient selection

Recently, a chemoprevention biomarker study was done in proven or probable HNPCC patients at the University Medical Center Groningen (20). Proven patients were carriers of a mutation in one of the mismatch repair genes (*hMLH1, hMSH2, hMSH6*). Probable HNPCC patients had a family history meeting the revised Amsterdam criteria (21) and a medical history of an HNPCC-associated cancer, a colorectal adenoma at an early age (<40 years), or an adenoma with advanced neoplastic characteristics. Individuals with prior colorectal surgery were enrolled when the estimated length of the remaining colon exceeded 50% of the original length. In this randomized double-blind placebo-controlled crossover study, patients were assigned to receive sulindac 150 mg orally twice daily or an identically appearing placebo for 4 weeks. Both were produced by the Pharmacy Department of the University Medical Center. After a washout period of 4 weeks, patients crossed over to the alternative treatment for another 4 weeks. Full colonoscopy was done at 4 and 12 weeks. Biopsies were taken of macroscopically normal mucosa with a standard biopsy forceps at four locations: ascending, transverse, and sigmoid colon, and rectum. Samples were formalin fixed, embedded in paraffin, and coded to disguise the subjects' treatment assignment. The local medical ethical committee approved the study. Reasons for exclusion from the study were use of a NSAIDs in the 3 months before the study, pregnancy, or a history of peptic ulcer disease or gastrointestinal bleeding.

For the present study, sufficient residual material was available from 18 patients (12 men, 6 women; mean age, 44.6 years). Nine of these patients were proven carriers of a mutation in the mismatch repair gene *hMSH2* (n = 6) or *hMLH1* (n = 3). Samples from FAP patients were obtained from a study in which FAP patients had been treated with sulindac 150 mg twice daily during 9 months, as described previously (2, 22). From six

patients, tissue sections were available from normal-appearing rectal mucosa before and after 6 months of treatment. These six patients had adenomas at baseline and showed regression of adenomas after treatment with sulindac.

Immunohistochemistry for apoptosis, DR4, DR5, β-Catenin, and p21

For immunohistochemistry, 3 μm thick sections were cut from paraffin blocks and deparaffinized in xylene. Apoptosis was determined using the murine monoclonal antibody M30 (Boehringer Mannheim, Mannheim, Germany) directed against cleaved cytokeratin-18 that is expressed during early apoptosis (23). Staining procedures for M30, DR4, and DR5 were done as previously described (23, 24). For β-catenin staining (1:1,000; clone 14, Transduction Laboratories, Lexington, KY), antigen retrieval was carried out by microwave treatment for 8 minutes at 700 W in 0.01 mol/L citrate buffer (pH 6.0). For p21 staining (1:50; clone WAF1, Oncogene Research, Darmstadt, Germany), antigen retrieval was done by heating slides thrice for 5 minutes at 115°C with 5-minute cooling in between in maleate buffer in a preheated autoclave (Presto deluxe, Presto, Eclaire, WI). After blocking of endogenous peroxidase with 0.3% hydrogen peroxide for 30 minutes and incubation with avidin and biotin blocking solutions (Vector Laboratories, Burlingame, CA), primary antibodies were applied for 1 hour at room temperature. After washing with PBS, slides were incubated with appropriate secondary and tertiary antibodies. Slides were counterstained with hematoxylin. As negative controls, slides were stained in absence of the primary antibody. As positive controls, sections of normal human liver (DR4 and DR5) and colorectal cancer (β-catenin, p21, and M30) were included. For each antibody, slides were stained in one batch.

Evaluation of staining

Slides were independently evaluated by light microscopy by two investigators in a coded fashion. For M30 and p21, positive cells were expressed as percentage of the total number of cells counted (apoptotic and p21 indices, respectively). Only complete longitudinal crypts and at least 500 cells were counted. Intensity of DR4, DR5, and β-

catenin staining was semiquantitatively graded using a scale from 1 to 3 (1, weak staining; 2, moderate staining; 3, intense staining). For β-catenin, staining was separately recorded as membranous, cytoplasmic, or nuclear. To assess changes in staining intensity as a consequence of treatment, intensities were compared in paired slides and scored as increased, decreased, or unchanged. When the observers' scores differed, cases were re-evaluated using a multiheaded microscope and the final grade was reached by consensus.

Statistics

For statistical assessment of changes in apoptotic indices, p21 indices, and cumulative DR4, DR5, and β-catenin expression scores following sulindac treatment versus placebo, the Wilcoxon rank sum test for paired samples was used. Changes in distribution of staining intensities of DR4, DR5, and β-catenin were assessed using chi-square tests. To determine differences between various colonic regions in HNPCC patients, Mann-Whitney tests for continuous variables and chi-square tests for discontinuous variables were conducted. Differences between proven and probable HNPCC patients were assessed using Mann-Whitney test for continuous variables and chi-square tests for discontinuous variables. Reported *P* values were two tailed and significance was assumed at $P < 0.05$. SPSS for Windows software (SPSS, Inc., Chicago, IL) was used for all statistical analyses.

Results

To assess changes in apoptosis, DR4, DR5, β-catenin, and p21 expression following placebo and sulindac, samples were analyzed pairwise, comparing staining results in biopsies from the same patient in the same colonic region. The analysis of sample pairs was hampered by the problem of limited availability of material. In case one in a pair of samples contained insufficient material to allow evaluation, it meant that these samples were not evaluated. Not all biopsies obtained in HNPCC patients were of sufficient quality, limiting the number of sample pairs analyzed to 55 for apoptotic

indices, 64 for DR4, 67 for DR5, 48 for β-catenin, and 63 for p21 expression. The number of sample pairs analyzed from FAP patients was six for all staining procedures.

Apoptosis

When comparing cumulative apoptotic indices between placebo and sulindac treatment (in HNPCC) and between pretreatment and posttreatment with sulindac (in FAP), no statistically significant differences were observed (Table 1, left panel). Given the predilection for the proximal colon in the development of colorectal neoplasia in HNPCC, apoptotic indices were compared in different colonic regions in HNPCC patients. For each region, apoptotic indices were not significantly different following sulindac compared with placebo although in biopsies from the proximal colon there was a trend towards lowering of apoptotic indices following sulindac.

DR4, DR5, and β-catenin expression following placebo (HNPCC) and at baseline (FAP)

In all patient samples, cytoplasmic staining of DR4 and DR5 was observed. For DR4, the immunoreactivity of epithelial cells increased gradually from the crypt base to the luminal surface. DR5 immunoreactivity was seen along the entire crypt axis. β-catenin expression was membranous in all investigated samples (i.e., no cases of cytoplasmic or nuclear staining were seen). In HNPCC patients, DR4, DR5, and β-catenin staining intensities were similar in all four investigated regions of the colon. No differences were seen between proven carriers of MLH1 or MSH2 gene mutations and patients without an established mutation. Also, no differences in expression patterns were observed between MLH1 and MSH2 mutation carriers.

Changes in DR4, DR5, and β-catenin expression following sulindac (HNPCC and FAP)

Alterations in expression patterns of DR4, DR5, and β-catenin were analyzed, studying the distribution of staining intensities and changes in absolute and cumulative staining intensity scores. To assess whether changes in staining intensities were consistent in

Table 1. Mean apoptotic and p21 indices in sample pairs from normal colon mucosa following sulindac compared with placebo (HNPCC) and baseline values (FAP).

	n	Apoptotic index* (%)		*n*	P21* (%)	
HNPCC		**Placebo**	**Sulindac**		**Placebo**	**Sulindac**
Cumulative	55	0.76 ± 0.12	0.70 ± 0.09	63	38.1 ± 1.1	39.7 ± 0.9
Ascendens	14	0.81 ± 0.13	0.67 ± 0.18	16	36.9 ± 3.0	42.7 ± 2.4
Transverse	16	1.03 ± 0.39	0.69 ± 0.11	17	38.2 ± 2.2	38.7 ± 1.2
Sigmoid	14	0.56 ± 0.11	0.94 ± 0.25	17	36.8 ± 2.5	36.2 ± 1.2
Rectum	11	0.57 ± 0.11	0.43 ± 0.11	13	40.4 ± 2.2	41.9 ± 2.0
FAP		**Baseline**	**Sulindac**		**Baseline**	**Sulindac**
Rectum	6	0.88 ± 0.25	0.38 ± 0.31	6	17.7 ± 0.5	31.6 ± 12.4

*Data expressed as mean ± SE.

HNPCC patients, cumulative scores were calculated for each patient by adding the respective intensity scores in the samples from different parts of the colon. Cumulative scores were calculated when at least two sample pairs per patient were available. Table 2 summarizes changes in the distribution of staining intensities of DR4, DR5, and β-catenin expression following sulindac compared with placebo (HNPCC) and baseline (FAP). For DR4 and DR5, staining scores were similarly distributed following sulindac compared with placebo in HNPCC patients and compared with baseline values in FAP patients. For β-catenin, staining scores were distributed differently following sulindac compared with placebo in HNPCC ($P < 0.001$) and compared with baseline values in FAP samples ($P < 0.01$; Table 2), with lower scores in both patient groups after sulindac. In sample pairs, the intensity scores of DR4 staining were not consistently different following sulindac compared with placebo in HNPCC: higher in 26/64 pairs, lower in 27/64 pairs, and unchanged in 11/64 pairs (not significant). For DR5, similar results were obtained: higher in 19/67, lower in 20/67, and unchanged in 28/67 pairs (not significant). The intensity scores of membranous β-catenin staining following sulindac compared with placebo were higher in 7/48, lower in 26/48, and unchanged in 15/48 pairs ($P < 0.05$). In paired FAP samples, DR4 and DR5 staining intensities were similar between baseline and sulindac in all six samples. For membranous β-catenin,

staining intensities were lower following sulindac in three of six and unchanged in three of six FAP pairs (not significant). In cases with lower staining intensity of membranous β-catenin following sulindac, no apparent increase in cytoplasmic or nuclear staining was seen. With respect to cumulative staining intensity scores in HNPCC, scores were similar for DR4 and DR5 following placebo and sulindac (data not shown). However, for β-catenin, cumulative intensity scores were significantly lower following sulindac compared with placebo ($P < 0.01$; Fig. 1).

Table 2. Distribution of DR4, DR5, and B-catenin staining intensities in sample pairs from normal colon mucosa following sulindac, compared with placebo (HNPCC) and baseline values (FAP).

		HNPCC		FAP	
	Staining score*	Placebo	Sulindac	Baseline	Sulindac
		n/n tested (%)†		n/n tested (%)	
DR4	1	5/64 (8%)	6/64 (9%)	0/6	1/6
	2	31/64 (48%)	32/64 (50%)	5/6	4/6
	3	28/64 (44%)	26/64 (41%)	1/6	1/6
DR5	1	10/67 (15%)	9/67 (13%)	4/6	3/6
	2	36/67 (54%)	39/67 (58%)	2/6	3/6
	3	21/67 (31%)	19/67 (28%)	0/6	0/6
β-catenin	1	4/48 (8%)	9/48 (19%)‡	0/6	2/6 $
	2	24/48 (50%)	33/48 (69%)	4/6	3/6
	3	20/48 (42%)	6/48 (12%)	2/6	1/6

*Assessed as described in the Materials and Methods section.

†Number of samples investigated varied in patient groups as a consequence of limited availability of slides.

‡Distribution of staining intensities of h-catenin in HNPCC following placebo versus sulindac, $P < 0.001$.

$Distribution of staining intensities of β-catenin in FAP at baseline versus sulindac, $P < 0.01$.

p21

Mean percentages of p21-positive cells following placebo and sulindac are shown in Table 1 (right). After placebo, p21 indices were comparable in different colon regions in HNPCC patients. p21 indices were not significantly different between HNPCC and FAP patients. Following sulindac, p21 indices were similar compared with placebo (HNPCC) and baseline (FAP) values.

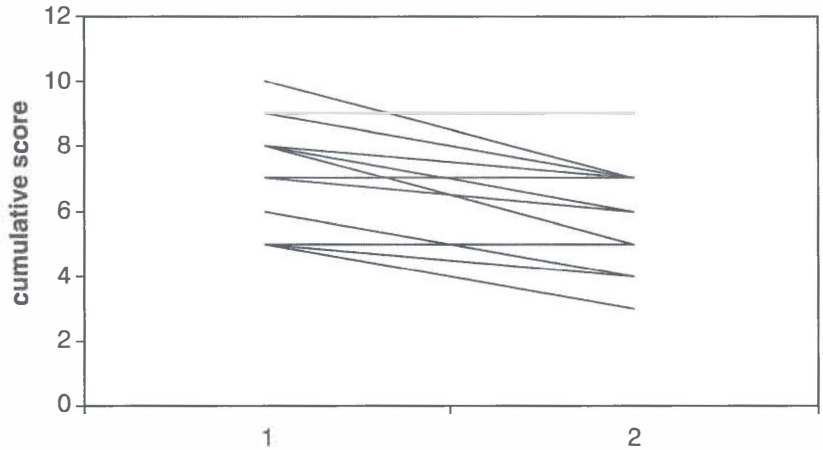


Figure 1. Cumulative β-catenin intensity scores in paired samples from HNPCC patients (n = 12) following placebo (1) and sulindac (2).

Discussion

The efficacy of chemopreventive agents in the colorectum is routinely assessed by measuring one or more end points: biomarker modulation in the at-risk mucosa, adenoma regression, adenoma suppression, or adenoma prevention (25). Our biomarker modulation study evaluated changes in apoptosis and expression of DR4, DR5, β-catenin, and p21 occurring in normal-appearing mucosa following treatment with sulindac in HNPCC and FAP patients. Although, in general, few conclusions can be drawn from biomarker studies, they provide an opportunity to identify mechanisms of action of chemopreventive agents. In particular, quantitative measurements of apoptosis are considered a sensitive index of the biological effects of nonsteroidal anti-inflammatory drugs (6). Whereas several biomarker modulation studies are available in FAP patients, our placebo-controlled crossover study is one of only a few in HNPCC patients. We found that sulindac did not alter the apoptotic index in normal colorectal mucosa from HNPCC and FAP patients compared with placebo (HNPCC) or baseline (FAP). As anticipated from these null results, no changes were seen in expression of the death receptors DR4 and DR5. However, reduced membranous β-catenin expression patterns were observed following sulindac in both patient groups,

suggesting an inhibiting effect of sulindac on the APC-β-catenin-Wnt pathway. Sulindac is one of the most extensively studied NSAIDs in the setting of chemoprevention of colorectal cancer (7). An important mechanism behind the chemopreventive effect of sulindac seems to be the induction of apoptosis (7). Sulindac is a prodrug that is converted into sulindac sulfide and then sulindac sulfone by colonic bacteria (26). In vitro, both metabolites induce apoptosis in colon cancer cells (7,11,27), including mismatch repair–deficient cells (28). In APC^{Min} mice, a mouse model of FAP, sulindac had an antitumor effect and was associated with induction of apoptosis (29). Also in a mismatch repair–deficient APC^{min} mouse model, carrying genetic features of both FAP and HNPCC, sulindac inhibited intestinal adenoma development (30). Whether this effect was mediated by induction of apoptosis was not studied. In normal rectal mucosa of FAP patients with adenomas, an increase or change of apoptosis has been observed following sulindac therapy (8,31). We did not find a significant effect on apoptosis in our FAP material, but this may be due to the limited number of cases. Interestingly, in presymptomatic, phenotypically unaffected FAP patients, no changes in apoptosis were seen on sulindac treatment (32). In accordance with these data, sulindac did not have a preventive effect on the development of adenomas in these phenotypically unaffected patients (33). There is no data on the efficacy of chemoprevention in HNPCC patients, although studies are ongoing (6, 34). Taken together, the chemopreventive action of sulindac in FAP patients seems to be mediated by induction of apoptosis, but limited to the stage when adenomas have already developed.

Recent studies have suggested that β-catenin is a target for the chemopreventive action of NSAIDs (16, 35, 36). In vitro, NSAIDs, including sulindac, prevented nuclear accumulation of β-catenin (16, 35). Oncogenic activation of the Wnt signaling pathway resulting in nuclear translocation of β-catenin is considered critical for the initiation in intestinal epithelial neoplastic transformation (37). A recent report reveals that adenomas from FAP patients showed less nuclear β-catenin staining after sulindac treatment (15). Similar results were obtained in APC^{Min} mice, in normal intestinal mucosa (38) as well as in adenomas (39). Our results in normal colon mucosa, with a

reduction in membranous expression of β-catenin following sulindac, are consistent with these data. Whether this phenomenon is limited to subjects with a predisposition for colorectal adenoma development or also applies to the general population remains unknown. Finally, we assessed whether changes in β-catenin expression were associated with alterations in p21 expression. Recent data indicated that active Wnt signaling decreases p21 concentrations, preventing cells from entering G1 arrest or differentiation, thereby allowing cells to proliferate (40). In a previous study of three patients treated with sulindac, p21 expression increased in two compared with pretreatment values in rectal biopsy specimens (41). Our results in a larger patient group do not confirm these data. Although we recently postulated that sulindac could mediate its effect on intestinal adenoma formation by modifying p21 expression (19), the present study does not support such a mechanism.

In summary, in normal colorectal mucosa from HNPCC and FAP patients, sulindac had an inhibiting effect on β-catenin expression without affecting apoptotic indices and DR4, DR5, and p21 expression. Our data provide further support for inhibition of the Wnt signaling as a contributing mechanism of chemoprevention by sulindac. Whether this effect is universal or limited to patients genetically predisposed to colorectal cancer remains unclear.

References

1. Jass JR, Whitehall VL, Young J, et al. Emerging concepts in colorectal neoplasia. Gastroenterology 2002;123:862 – 76.
2. Giardiello FM, Hamilton SR, Krush AJ, et al. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N Engl J Med 1993;328:1313 – 6.
3. Nugent KP, Farmer KC, Spigelman AD, et al. Randomized controlled trial of the effect of sulindac on duodenal and rectal polyposis and cell proliferation in patients with familial adenomatous polyposis. Br J Surg 1993;80:1618 – 9.
4. Winde G, Schmid KW, Brandt B, et al. Clinical and genomic influence of sulindac on rectal mucosa in familial adenomatous polyposis. Dis Colon Rectum 1997;40:1156 – 68.
5. Steinbach G, Lynch PM, Phillips RK, et al. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med 2000;342:1946 – 52.
6. Thun MJ, Henley SJ, Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. J Natl Cancer Inst 2002;94:252 – 66.
7. Chan TA. Nonsteroidal anti-inflammatory drugs, apoptosis, and coloncancer chemoprevention. Lancet Oncol 2002;3:166 – 74.
8. Pasricha PJ, Bedi A, O'Connor K, et al. The effects of sulindac on colorectal proliferation and apoptosis in familial adenomatous polyposis. Gastroenterology 1995;109:994 – 8.
9. Rigas B, Shiff SJ. Nonsteroidal anti-inflammatory drugs and the induction of apoptosis in colon cells: evidence for PHS-dependent and PHS-independent mechanisms. Apoptosis 1999;4:373 – 81.
10. Ashkenazi A, Dixit VM. Apoptosis control by death and decoy receptors. Curr Opin Cell Biol 1999;11:255 – 60.
11. Zhang L, Yu J, Park BH, et al. Role of BAX in the apoptotic response to anticancer agents. Science 2000;290:989 – 92.
12. He Q, Luo X, Huang Y, et al. Apo2L/TRAIL differentially modulates the apoptotic effects of sulindac and a COX-2 selective non-steroidal antiinflammatory agent in Bax-deficient cells. Oncogene 2002;21:6032 – 40.
13. Huang Y, He Q, Hillman MJ, et al. Sulindac sulfide-induced apoptosis involves death receptor 5 and the caspase 8-dependent pathway in human colon and prostate cancer cells. Cancer Res 2001;61:6918 – 24.
14. Bienz M, Clevers H. Linking colorectal cancer to Wnt signaling. Cell 2000;103:311 – 20.
15. Boon EM, Keller JJ, Wormhoudt TA, et al. Sulindac targets nuclear β-catenin accumulation and Wnt signalling in adenomas of patients with familial adenomatous polyposis and in human colorectal cancer cell lines. Br J Cancer 2004;90:224 – 9.
16. Gardner SH, Hawcroft G, Hull MA. Effect of nonsteroidal anti-inflammatory drugs on β-catenin protein levels and catenin-related transcription in human colorectal cancer cells. Br J Cancer 2004;91:153 – 63.
17. Rice PL, Kelloff J, Sullivan H, et al. Sulindac metabolites induce caspase- and proteasome-dependent degradation of β-catenin protein in human colon cancer cells. Mol Cancer Ther 2003;2:885 – 92.
18. Orner GA, Dashwood WM, Blum CA, et al. Suppression of tumorigenesis in the Apc(min) mouse: down-regulation of β-catenin signaling by a combination of tea plus sulindac. Carcinogenesis 2003;24:263 – 7.
19. Huls G, Koornstra JJ, Kleibeuker JH. Non-steroidal anti-inflammatory drugs and molecular carcinogenesis of colorectal carcinomas. Lancet 2003;362: 230 – 2.
20. Rijcken FE, Hollema H, van der Sluis T, Boersma-van Ek W, Kleibeuker JH. Sulindac increases epithelial cell proliferative activity in the proximal colon of HNPCC patients. Eur J Gastroenterol Hepatol 2005; 17:A56 – 7.
21. Vasen HF, Watson P, Mecklin JP, et al. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology 1999;116: 1453 – 6.
22. Giardiello FM, Offerhaus JA, Tersmette AC, et al. Sulindac induced regression of colorectal adenomas in familial adenomatous polyposis: evaluation of predictive factors. Gut 1996;38:578 – 81.

23. Koornstra JJ, Rijcken FE, De Jong S, et al. Assessment of apoptosis by M30 immunoreactivity and the correlation with morphological criteria in normal colorectal mucosa, adenomas and carcinomas. Histopathology 2004;44:9 – 17.
24. Koornstra JJ, Kleibeuker JH, van Geelen CMM, et al. Expression of TRAIL (TNF-related apoptosis-inducing ligand) and its receptors in normal colonic mucosa, adenomas, and carcinomas. J Pathol 2003;200:327 – 35.
25. Hawk E, Lubet R, Limburg P. Chemoprevention in hereditary colorectal cancer syndromes. Cancer 1999;86:2551 – 63.
26. Rao CV, Rivenson A, Simi B, et al. Chemoprevention of colon carcinogenesis by sulindac, a nonsteroidal anti-inflammatory agent. Cancer Res 1995;55:1464 – 2.
27. Shiff SJ, Qiao L, Tsai LL, et al. Sulindac sulfide, an aspirin-like compound, inhibits proliferation, causes cell cycle quiescence, and induces apoptosis in HT-9 colon adenocarcinoma cells. J Clin Invest 1995;96:491 – 503.
28. Ruschoff J, Wallinger S, Dietmaier W, et al. Aspirin suppresses the mutator phenotype associated with hereditary nonpolyposis colorectal cancer by genetic selection. Proc Natl Acad Sci U S A 1998;95:11301 – 6.
29. Mahmoud NN, Boolbol SK, Dannenberg AJ, et al. The sulfide metabolite of sulindac prevents tumors and restores enterocyte apoptosis in a murine model of familial adenomatous polyposis. Carcinogenesis 1998;19:87 – 91.
30. Lal G, Ash C, Hay K, et al. Suppression of intestinal polyps in Msh2- deficient and non-Msh2-deficient multiple intestinal neoplasia mice by a specific cyclooxygenase-2 inhibitor and by a dual cyclooxygenase-1/2 inhibitor. Cancer Res 2001;61:6131 – 6.
31. Keller JJ, Offerhaus GJ, Polak M, et al. Rectal epithelial apoptosis in familial adenomatous polyposis patients treated with sulindac. Gut 1999;45:822 – 8.
32. Keller JJ, Offerhaus GJ, Hylind LM, et al. Rectal epithelial apoptosis does not predict response to sulindac treatment or polyp development in presymptomatic familial adenomatous polyposis patients. Cancer Epidemiol Biomarkers Prev 2002;11:670 – 1.
33. Giardiello FM, Yang VW, Hylind LM, et al. Primary chemoprevention of familial adenomatous polyposis with sulindac. N Engl J Med 2002;346:1054 – 9.
34. Annie Yu HJ, Lin KM, Ota DM, et al. Hereditary nonpolyposis colorectal cancer: preventive management. Cancer Treat Rev 2003;29:461 – 70.
35. Hawcroft G, D'Amico M, Albanese C, et al. Indomethacin induces differential expression of beta-catenin, gamma-catenin and T-cell factor target genes in human colorectal cancer cells. Carcinogenesis 2002;23:107 – 14.
36. Smith ML, Hawcroft G, Hull MA. The effect of non-steroidal antiinflammatory drugs on human colorectal cancer cells: evidence of different mechanisms of action. Eur J Cancer 2000;36:664 – 74.
37. Giles RH, van Es JH, Clevers H. Caught up in a Wnt storm: Wnt signaling in cancer. Biochim Biophys Acta 2003;1653:1 – 24.
38. Mahmoud NN, Boolbol SK, Bilinski RT, et al. APC gene mutation is associated with a dominant-negative effect upon intestinal cell migration. Cancer Res 1997;57:5045 – 50.
39. McEntee MF, Chiu CH, Whelan J. Relationship of β-catenin and Bcl-2 expression to sulindac-induced regression of intestinal tumors in Min mice. Carcinogenesis 1999;20:635 – 40.
40. van de Wetering M, Sancho E, Verweij C, et al. The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241 – 50.
41. Yang W, Velcich A, Mariadason J, et al. p21(WAF1/cip1) is an important determinant of intestinal cell response to sulindac in vitro and in vivo. Cancer Res 2001;61:6297 – 302.

CHAPTER 7

Serial ^{111}In-mapatumumab SPECT scan in cancer patients treated with mapatumumab, gemcitabine and cisplatin

CNAM Oldenhuis[1], ECF Dijkers[2,3], AH. Brouwers[3], MW den Hollander[1] J Bart[4], NL Fox[5], JL Klein[5], JGW Kosterink[2], JR de Jong[3], JA Gietema[1], RA Dierckx[3], S de Jong[1], MN Lub-de Hooge[2,3], EGE de Vries[1].

Departments of Medical Oncology[1], Hospital and Clinical Pharmacy[2], Nuclear Medicine and Molecular Imaging[3] and Pathology[4], University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; Human Genome Sciences[5], Rockville, MD, USA.

Submitted.

Abstract

Purpose: To visualize tumor lesions by serial ^{111}In-mapatumumab scintigraphy and determine ^{111}In-mapatumumab pharmacokinetics in patients with advanced solid tumors at the start and during treatment with the TRAIL-R1 targeting antibody mapatumumab plus gemcitabine and cisplatin.

Experimental design: Patients with advanced solid tumors received mapatumumab 20 mg/kg iv and cisplatin 80 mg/m^2 iv on day 1 and gemcitabine 1250 mg/m^2 iv on days 1 and 8, every 21 days. Patients received 150 MBq ^{111}In-mapatumumab in cycle 1 and cycle 3. Thirty minutes and at day 1, 3 and 6 after the first and second tracer injection planar whole body imaging and single-photon emission computed tomography (SPECT) were performed. SPECT images were fused with CT. TRAIL-R1 tumor expression was assessed immunohistochemically. Pharmacokinetic analysis of ^{111}In-mapatumumab was executed in both cycles.

Results: In 5 of the 12 patients, 11 out of 18 tumor lesions known by CT were visualized with ^{111}In-mapatumumab SPECT. A large heterogeneity was seen in ^{111}In-mapatumumab uptake between these patients. Two melanoma patients showed remarkable intense tracer uptake. In 3 patients all lesions were visualized. SPECT results in cycle 3 were comparable to the first SPECT series. Three of the 4 patients having positive ^{111}In-mapatumumab scintigraphy and tumor tissue available showed at least low cytoplasmic TRAIL-R1 expression. Intensity of staining did not correlate with positive ^{111}In-mapatumumab scintigraphy or tumor response to treatment.

Conclusions: The ^{111}In-mapatumumab tumor uptake in this group of patients was variable and the value of tumor imaging in upfront patient selection for mapatumumab-based treatment needs further evaluation.

Introduction

Achieving tumor cell death is an ultimate goal in anticancer treatment. The naturally occurring Tumor Necrosis Factor (TNF)-Related Apoptosis Inducing Ligand (TRAIL) induces apoptosis via activation of the TRAIL receptors (TRAIL-R1 and TRAIL-R2) are present on a broad range of tumor cells at variable expression levels. Moreover, TRAIL induces apoptosis in cancer cells, but not normal cells. These findings have raised interest in inducing tumor apoptosis via TRAIL-R1 and TRAIL-R2 targeting.

Mapatumumab (HGS-ETR1, TRM-1) is a fully human TRAIL-R1 agonistic monoclonal antibody (mAb). Single agent mapatumumab showed no major toxicities in two phase I studies in patients with advanced solid tumors or non-Hodgkin's lymphomas (1,2). The mean terminal elimination half-life of mapatumumab ranged from 14 to 28 days (1,2). Three phase II single agent studies have been conducted in patients with non-small cell lung cancer, non-Hodgkin's lymphoma or colorectal cancer, respectively (3-5). In the non-small cell lung cancer and colorectal cancer studies, the best observed response was stable disease and in the non-Hodgkin's lymphoma study two complete and one partial tumor responses were reported.

Mapatumumab combined with gemcitabine and cisplatin resulted in increased cytotoxicity in human tumor cell lines and mouse xenograft models. In a phase I study patients with solid malignancies were treated with this combination (6). This combination with maptumumab up to 30 mg/kg every 3 weeks was well tolerated and safe. No alterations in the pharmacokinetic profiles of the drugs were observed. Partial responses were observed in 12 patients across dose levels and stable disease was seen in 25 patients.

At the moment little is known about the tissue biodistribution of mapatumumab. It is unknown whether mapatumumab reaches the tumor in sufficient levels to be effective against tumor cells. Interestingly, in mice bearing human colorectal tumor xenografts, TRAIL-R1 was upregulated by chemotherapy and antitumor activity was markedly enhanced (7). It is unknown whether upregulation of TRAIL-R1 in the tumor occurs in patients as a result of concomitant chemotherapy.

We hypothesized that radiolabeled mapatumumab can help to visualize high TRAIL-R1 expressing tumor lesions and support future selection of patients that may benefit from this treatment. We therefore developed indium-111 (^{111}In) radiolabeled mapatumumab suitable for clinical use. In high TRAIL-R1 expressing human xenografts in mice specific ^{111}In-mapatumumab uptake was shown (8).

The aim of this study was to evaluate tumor lesion visualization (targeting) by serial ^{111}In-mapatumumab scintigraphy in patients with advanced solid tumors at the start and during treatment with mapatumumab plus gemcitabine and cisplatin. Furthermore, the biodistribution of ^{111}In-mapatumumab was studied, including calculation of pharmacokinetic parameters.

Patients and Methods

Eligibility criteria

Eligibility criteria for this study were similar to inclusion criteria in the preceding phase I study (6). Eligibility criteria included: patients with histologically or cytologically confirmed advanced solid malignancies for whom no standard therapeutic options were available or for whom gemcitabine and cisplatin was considered an appropriate treatment; Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1; age ≥ 18 years; a life expectancy ≥ 3 months; adequate bone marrow, hepatic, and renal function; no other anti-cancer treatment within previous 3 weeks. Excluded were patients with known positive human immunodeficiency virus status; known chronic or acute viral hepatitis; clinical signs of brain metastases; hearing loss requiring the use of a hearing aid; neuropathy ≥ grade 2; myocardial infarction, cerebrovascular accident or ≥ NYHA class III congestive heart failure within 6 months.

The study was approved by the local medical ethical committee and is registered under trial number NTR2103. All patients provided written informed consent.

Treatment

Patients were treated as in the previous phase I study (6). Gemcitabine (Eli Lilly) 1250 mg/m^2 iv on days 1 and 8 and cisplatin (Pharmachemie) 80 mg/m^2 intravenously (iv) on

day 1 were administered. In addition, patients received mapatumumab (provided by Human Genome Sciences Inc) 20 mg/kg iv in 2 hours on day 2 of cycle 1 and 3 and on day 1 of cycles 2 and 4-6. Cycles were repeated every 3 weeks for a maximum of 6 cycles.

Chemotherapy doses were reduced according to the phase I study protocol (6): when treatment was delayed for > 1 week, in the case of severe toxicity (≥ grade 3 according to the National Cancer Institute Common Toxicity Criteria for Adverse Events version 3.0) considered to be at least possibly related to chemotherapy or for the patient's safety in the opinion of the investigator. Cycle delays up to 2 weeks were permitted for hematologic recovery. A baseline CT scan was performed within 28 days before the start of cycle 1. CT-response was assessed after 3 and 6 cycles by Response Evaluation Criteria in Solid Tumors (RECIST version 1.0) (9). In the absence of disease progression after 6 cycles, continuing mapatumumab monotherapy was allowed.

^{111}In-mapatumumab production and scintigraphy

Clinical grade ^{111}In-mapatumumab was produced as described earlier (10). In short, reconstituted mapatumumab was conjugated with the chelator 2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid (*p*-SCN-Bn-DTPA, Macrocyclics), purified by ultrafiltration and stored at -80°C. GMP-produced $^{111}InCl_3$ (Covidien) was used to radiolabel the conjugate. Quality control was performed to ensure antigen binding capacity (> 80%), stability and (radio)chemical purity (> 95%). Size-exclusion chromatography (SE-HPLC), thin-layer chromatography (TLC) and BIAcore analysis were performed as described earlier (8, 10).

Patients received 150 MBq ^{111}In-mapatumumab (10 mg), iv 1.5 hours after the start of the unlabeled mapatumumab infusion in cycle 1. If, after entering the first 4 patients in the study, tumor lesions were not adequately visualized with this treatment scheme, possibly due to TRAIL-R1 saturation by therapeutic mapatumumab dose, 150 MBq ^{111}In-mapatumumab would be administered 7 days before the unlabeled mapatumumab infusion in cycle 1. On day 2, cycle 3, the 150 MBq ^{111}In-mapatumumab was administered 1.5 hours following the start of the therapeutic dose mapatumumab

for both imaging schemes. All patients were monitored for infusion related reactions the first 4 hours after mapatumumab infusion.

Planar whole body imaging and single-photon emission computed tomography (SPECT) was performed at 30 minutes and on days 1, 3 and 6 after the first and second tracer injection. Planar whole body imaging was performed using a two-headed gamma-camera (Ecam or MultiSpect 2, both Siemens), equipped with parallel-hole medium-energy all purpose collimators, at a scan speed of 10 cm/minute (at 30 minutes and 1 day post-injection) or 5 cm/minute (at 3 and 6 days post-injection) and stored digitally in a 256x1024 matrix. SPECT images were acquired of pre-defined tumor regions, using 180 degrees of sampling with 32 projections per head, 45 seconds acquisition time per projection and a 128 x 128 matrix size.

Image and data analysis

Non-scatter corrected SPECT images were reconstructed iteratively with an ordered subset expectation maximization algorithm (8 iterations, 16 subsets). A 9.00 mm Gaussian filter was applied. Whole body and SPECT reconstruction images were analyzed by two investigators (AB and CO) for ^{111}In-mapatumumab uptake in possible tumor lesions. SPECT images were fused with conventional computed tomography (CT) images (obtained before start and after 3 cycles of treatment for response assessment) to validate regions of increased tracer uptake as tumor lesions, using a LEONARDO e.soft workstation (Siemens).

Dosimetry

The radiation-absorbed whole body and organ dose was estimated using the conjugated views counting technique with partial background subtraction and correction for attenuation and physical decay, as described previously (11, 12). Briefly, regions of interest (ROI) were drawn on the anterior and posterior whole body images of organs of interest that showed tracer uptake. Residence times were calculated using the SPRIND software package (13). Subsequently, OLINDA software was used to

calculate the organ radiation-absorbed doses and the effective dose, according to ICRP60 (14).

^{111}In-mapatumumab uptake in tumor lesions was assessed using the day 3 scan which showed the best image quality. 3D volumes of interest (VOIs) were drawn around tumor lesions and normal muscle in the fused SPECT/CT images. ^{111}In-mapatumumab accumulation was quantified as the mean and maximum counts per voxel, using a threshold of 40% of the maximum value. AMIDE Medical Image Data Examiner software (version 0.9.2, Stanford University (15)) was used as described previously (16). Tumor to normal muscle tissue ratios were calculated, to allow inter patient comparison.

^{111}In-mapatumumab pharmacokinetics

Venous blood samples in heparin collection tubes were obtained prior to, after 10 and 30 minutes, 1 and 4 hours and 1, 3, 6 and 13 days following the first and second ^{111}In-mapatumumab injection. Urine (24 hours) was collected during the first 4 days after tracer injection. Total radioactivity in the whole blood, plasma, and urine samples was determined using a calibrated gamma counter (well type LKB-1282-Compu-gamma system, LKB Wallac).

Pharmacokinetic parameters were derived using the Bayesian ITS module (KINPOP) of MW/PHARM (version 3.50, MediWare) for ^{111}In-mapatumumab blood clearance. The data from plasma, blood and urine were analyzed in a 1-, 2- and 3-compartment model to determine the best fit. ^{111}In-mapatumumab blood clearance was calculated using non-linear regression analysis. Based on the blood curves, the area under the curve (AUC) half life ($T_{½}$) during distribution (α) and elimination (β) phase, total clearance (CL), volume of distribution (V1) and steady-state volume (V_{ss}) were determined.

Statistics

Associations between parameters were assessed with Pearson correlation coefficients. Differences between pharmacokinetic data were calculated using the non-parametric

Mann Whitney U-test in SPSS 16.0 (SPSS Inc.). Two-sided *P*-values ≤ 0.05 were considered statistically significant.

Immunohistochemistry

For immunohistochemistry, pretreatment tumor tissue was used. Slides of 3 µm were cut from paraffin blocks and stained immunohistochemically for TRAIL-R1 as described previously (17). They were scored for TRAIL-R1 staining intensity into four categories (no staining, low, moderate, and strong) by two investigators (CO, not blinded for clinical outcome, and JB, blinded for clinical outcome). Sections of normal human liver served as positive controls.

Results

Patients

Twelve patients were enrolled. Patient characteristics are summarized in Table 1. One patient was taken off study after one treatment cycle because of severe nausea and vomiting. Two patients discontinued the study after 1 and 2 cycles because of early disease progression. These 3 patients therefore did not undergo the second imaging series. One melanoma patient was treated with 22 cycles before progressive disease was observed. The best response in this patient was stable disease.

The treatment was in general well tolerated. The observed toxicity was comparable to the previous phase I study and consisted mainly of nausea, vomiting, and fatigue (6). Three patients discontinued cisplatin prematurely, two because of nausea and vomiting, despite optimization of antiemetic treatment after 2 and 5 cycles respectively and in one patient asymptomatic decrease in glomerular filtration rate occurred after the day 1 treatment of cycle 1.

Of the 9 evaluable patients, 8 showed stable disease. One patient with pancreatic cancer had a confirmed partial response after 3 cycles.

Table 1. Patient characteristics.

Patient characteristics (n=12)	
Age (years)	
Median (range)	56 (47-70)
Sex	
Male	8
Female	4
Prior treatment	
Systemic	4
Radiotherapy	2
Tumor types	
Pancreatic adenocarcinoma	5
(Adeno)carcinoma of unknown primary	3
Non-small cell lung cancer	2
Melanoma	2
Number of treatment cycles	
Median (range)	5 (1-22)

^{111}In-mapatumumab scintigraphy

No infusion related anaphylactic reactions or other adverse events were seen upon ^{111}In-mapatumumab administration. Twelve patients underwent the first imaging series. Visual analysis of the SPECT scans showed retained ^{111}In-mapatumumab blood pool activity during the scan sequence in all patients (Figure 1). This was confirmed by the calculated long ^{111}In-mapatumumab elimination half-life of approximately 13 days (Table 2). In addition to the blood pool, highest ^{111}In-mapatumumab uptake was seen in liver, spleen, and kidneys. The ^{111}In-mapatumumab mean effective whole body dose determined in this study was 0.20 mSv/MBq (Table 3). The calculated radiation absorbed dose for normal organs did not differ significantly after the first and second ^{111}In-mapatumumab injection (Table 3).

The first 4 evaluable patients showed limited tumor visualization. Therefore, in the next patients, ^{111}In-mapatumumab was administered 7 days before the mapatumumab infusion in cycle 1.

Table 2. Pharmacokinetic parameters after 10 mg ^{111}In-Mapatumumab-injection (mean ± SD).

	AUC (h*mg/L)	T½,α (d)	T½,β (d)	CL (mL/d/kg)	V1 (mL/kg)	Vdss (mL/kg)	Urinary excretion (% ID/d)
N = 17	956.0 ± 314.4	1.0 ± 0.6	12.7 ± 4.2	4.3 ± 1.1	44.0 ± 6.8	70.2 ± 19.7	1.4 ± 0.01

Abbreviations: SD, standard deviation; AUC, area under plasma concentration-time curve; $T_{½,\alpha}$ and $T_{½,\beta}$, ^{111}In-mapatumumab half-life during distribution phase and elimination phase respectively; CL, total clearance; V1, volume of distribution for the central compartment; Vd_{ss}, volume of distribution at steady state; %ID/d, percentage of the injected dose/day.

Table 3. Radiation absorbed dose (organs) and effective dose (whole body).

	Scan 1 (n = 9) Mean ± SD (mGy/MBq)	Scan 2 (n = 7) Mean ± SD (mGy/MBq)
Heart	0.34 ± 0.04	0.34 ± 0.03
Kidneys	0.26 ± 0.03	0.23 ± 0.04
Liver	0.36 ± 0.07	0.36 ± 0.07
Lungs	0.25 ± 0.03	0.25 ± 0.02
Bone marrow	0.14 ± 0.01	0.14 ± 0.00
Spleen	0.32 ± 0.07	0.32 ± 0.09
Thyroid	0.14 ± 0.01	0.14 ± 0.00
Effective dose (whole body; mSv/MBq)	0.20 ± 0.01	0.21 ± 0.01

Abbreviation: SD, standard deviation.

The optimal time for the SPECT represents a balance between good tumor/non-tumor ratios and sufficient radioactive signal. The best tumor to background ratio was obtained 3 days post-tracer injection. The scans performed at day 6 were of inferior image quality as a consequence of insufficient counting statistics.

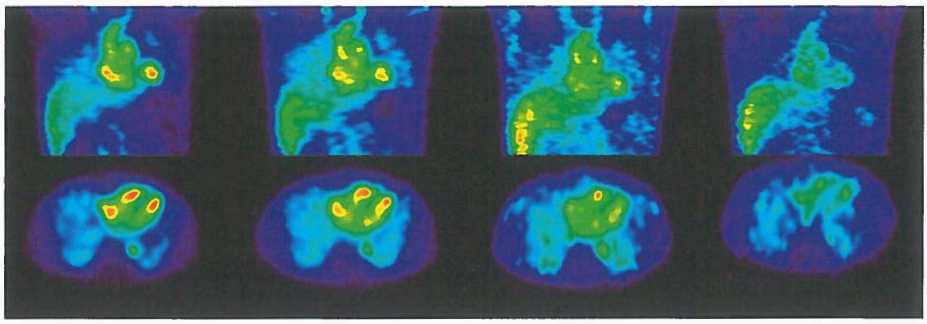


Figure 1. Coronal and transversal SPECT images of the chest and upper abdomen 30 minutes and 1, 3 and 6 days post-injection of ^{111}In-mapatumumab. Physiological ^{111}In-mapatumumab uptake is seen in predominantly the heart, the descending aorta and the liver. No tumor lesions are visible in these images.

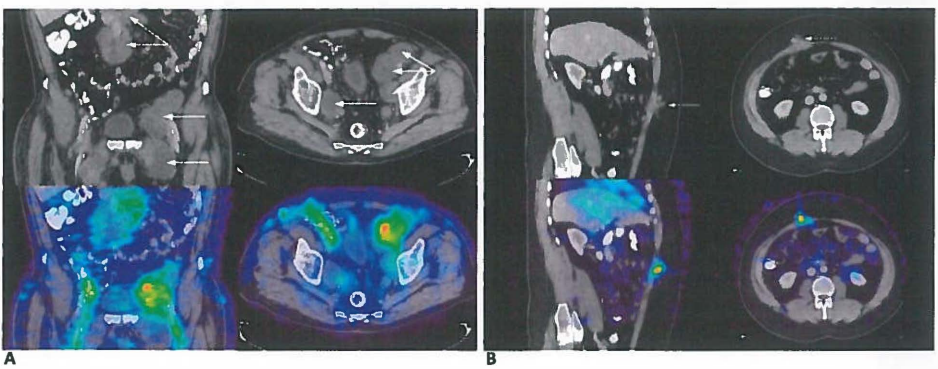


Figure 3. Coronal, transversal and sagittal CT images and ^{111}In-mapatumumab SPECT/CT fusion images of two melanoma patients, 3 days post-injection. (A) Tumor lesions in the upper abdomen and the left lower pelvic region show ^{111}In-mapatumumab uptake. (B) Intense ^{111}In-mapatumumab uptake in a tumor lesion in the abdominal wall. Arrows indicate tumor lesion.

In 2 of the 12 patients, strong ^{111}In-mapatumumab uptake in tumor lesions was detected when the whole body scintigraphy and SPECT reconstructions were visually analyzed. After fusion of CT and SPECT images, tumor lesions were visible in 3 additional patients. In these 5 patients, 11 out of 18 tumor lesions known by CT were imaged. Tracer uptake in these 11 lesions was variable.

The relation between mean and maximum ^{111}In-mapatumumab uptake was linear (r^2=1.00, P < .00001). Further analysis was therefore performed using the maximum uptake value, which is not influenced by individual drawing of VOIs. Quantification showed a ^{111}In-mapatumumab tumor to muscle ratio of median 3.3 (range, 2.2-8.0) (Figure 2).

Out of the 5 patients with ^{111}In-mapatumumab tumor uptake, 2 patients showed markedly intense visualization of metastases (Figure 3). Both patients were diagnosed with metastatic melanoma. One melanoma patient showed optimal ^{111}In-mapatumumab uptake in tumor lesions on the day-3 scan. In the other melanoma patient, the tumor lesion was already visible immediately after injection and continued to show on subsequent scans with the same intensity. In these 2 melanoma patients, 4 out of the 6 metastases positive in the first scan were also visualized in the second imaging series. One lesion not detected during the first imaging series, showed ^{111}In-mapatumumab tumor uptake during the second series. One of these melanoma patients discontinued the study after 3 cycles because of disease progression, the other melanoma patient was treated for 22 cycles before the disease progressed.

In the remaining 3 patients, visual analysis of images showed ^{111}In-mapatumumab tumor uptake in the identical lesions in both the first and second imaging series.

CT evaluation after the second scan in the 5 patients with ^{111}In-mapatumumab tumor uptake showed no difference in size of the individual tumor lesions if compared to baseline. No new tumor lesions were found with both CT scan and ^{111}In-mapatumumab scintigraphy at the time of the second scan (cycle 3).

There was no significant difference in ^{111}In-mapatumumab pharmacotherapeutic parameters between the first and second group of patients (P = 0.53) and between the first and second imaging series (P = 0.70). Data were therefore pooled (Table 2). In total 5 out of 22 datasets were excluded because of insufficient numbers of samples to allow reliable estimation of the pharmacokinetic parameters.

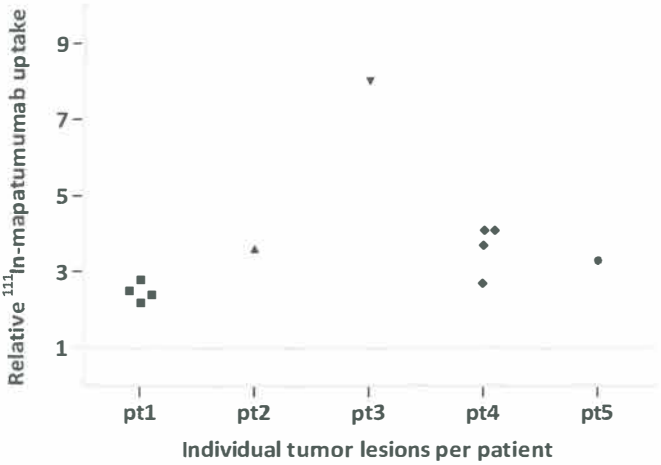


Figure 2. Tumor to normal muscle tissue ratios for the patients with ^{111}In-mapatumumab uptake in their tumor lesions (muscle uptake denoted as 1). Pt, patient.

Immunohistochemistry

Sufficient tumor tissue was available for immunohistochemical analysis in 9 patients. Pretreatment tumor tissue was used in 8 patients (obtained 2-9 months before enrollment). In 1 patient a tumor sample was obtained 20 months after completion of the study treatment. Eight patients showed at least low cytoplasmatic TRAIL-R1 expression in tumor cells. In 4 out of 5 patients with ^{111}In-mapatumumab uptake in tumor lesions, tumor tissue was available. Three of these patients showed at least low cytoplasmatic TRAIL-R1 expression in their tumor cells. In the remaining patient no TRAIL-R1 in tumor cells was detected. No membranous staining was seen. Intensity of staining did not correlate with positive ^{111}In-mapatumumab scintigraphy or tumor response to treatment.

Discussion

This study showed that ^{111}In-mapatumumab scintigraphy is feasible and can identify tumor lesions in advanced solid tumor patients. Tracer uptake in tumor lesions occurred in 5 out of 12 patients. In patients showing tumor uptake, not all tumor

lesions detected by CT scan were visualized. The most intense uptake was seen in 2 patients diagnosed with metastatic melanoma. This is the first proof that a TRAIL-R targeting monoclonal antibody can show preferential uptake in known sites of tumor in a subgroup of patients.

Mapatumumab was well-tolerated and can be administered repeatedly, in some cases for several years without apparent adverse effects (6). In the phase I studies, the maximum tolerated dose was not reached and the observed toxicity was mild when combined with chemotherapy. As single agent activity has been seen in lymphoma (5), it is possible that the drug might play a role in combination therapy. Proper patient selection of those that might benefit from this antibody is critical. Nuclear imaging may contribute to selecting the appropriate patients for the drug by visualizing specific uptake that accumulates in the tumor, as a potential predictor of efficacy of the therapy is sufficient drug reaching the target.

^{111}In-mapatumumab showed long blood pool circulation, indicated by the extended elimination half-life. This reflects a distribution which is generally seen for intact radiolabeled monoclonal antibodies (18). The long ^{111}In-mapatumumab circulation allows sufficient time for the ^{111}In-mapatumumab to accumulate in tissues during the time-frame of the imaging.

Large heterogeneity was seen in ^{111}In-mapatumumab uptake between the various patients with known metastases by CT. Given the heterogeneity of tracer uptake observed in this study, ^{111}In-mapatumumab scintigraphy could potentially identify patients that can benefit from mapatumumab-containing therapy.

Patients showing tumor tracer uptake in the first imaging series, also showed uptake in the second imaging series. This indicates that target saturation has not been reached, despite the fact the patients at that time had already received 3 therapeutic dosing cycles of 20 mg/kg each. This is likely explained by the high TRAIL-R1 receptor turnover that is seen after TRAIL-R1 endocytosis induced by TRAIL-R1 targeting (19). In our study we performed ^{111}In-mapatumumab scintigraphy before the start as well as during treatment. The absence of tumor saturation implies that both timing strategies can be used.

The ^{111}In-mapatumumab tumor uptake was highest in the 2 melanoma patients. A microarray study showed a higher TRAIL-R1 expression in 546 melanomas than in their benign nevi (20). Similar results were obtained in a study including 80 melanoma patients by immunohistochemical staining for TRAIL-R1 (21). In the current small study TRAIL-R1 expression was however not predictive for visualization of tumor lesions. The use of archival tumor tissue in this study may not represent the TRAIL-R1 expression status of the tumor at the time of imaging, as receptor status of tumors can change over time. A future larger study in patients with different tumor types will be needed to establish if high ^{111}In-mapatumumab tumor uptake is typical for patients with metastatic melanoma. The fact that one of the melanoma patients was treated for 22 cycles before the disease progressed may indicate that high ^{111}In-mapatumumab uptake may be predictive for at least disease stabilization following mapatumumab containing treatment.

In human colorectal cancer bearing mice treated with 60 mg/kg paclitaxel iv TRAIL-R1 expression levels increased 20-fold and ^{111}In-mapatumumab uptake was increased 1.4 fold (7). The current clinical trial with ^{111}In-mapatumumab scans before and after 3 cycles of chemotherapy did not indicate that this uptake increased following mapatumumab combined with gemcitabine and cisplatin.

^{111}In-mapatumumab differs from a number of other radiopharmaceuticals visualizing cell membrane receptors, as its target, TRAIL-R1, shows no real overexpression in tumor cells versus normal cells. This is in contrast to, for example, the folate receptor (up to 6 fold higher expression in ovarian cancer), or the human epidermal growth factor receptor 2 (HER2) (up to 100 fold higher expression in breast cancer) (22,23).

Because TRAIL-R1 shows no apparent overexpression, it can be anticipated that there is less driving force when compared with radiopharmaceuticals aimed at targets that show clear antigen overexpression, in terms of radiopharmaceutical accumulation. Because, in our study there is no clear correlation between immunohistochemically determined TRAIL-R1 expression and ^{111}In-mapatumumab tumor uptake, other factors determining drug tumor uptake might be involved including vascular density and intra-tumoral pressure. In addition, the tumor accumulation will be influenced by the rate of

TRAIL-R1 endocytosis upon mapatumumab antigen binding and renewal and recycling of the TRAIL-R1 membrane receptors (19). The current patient study shows much higher ^{111}In-mapatumumab tumor uptake in the two patients with metastatic melanoma than was seen in our animal study and higher than we anticipated.

SPECT was used to monitor mapatumumab tumor uptake. In future studies, mapatumumab could also be radiolabeled with the positron emission tomography (PET) isotope ^{89}Zr. The long-lived positron emitter ^{89}Zr has ideal physical characteristics for antibody imaging, such as a half-life comparable to ^{111}In of 3.27 days. Interesting results were seen in a recent clinical trial of ^{89}Zr-trastuzumab in patients with breast cancer and ^{89}Zr-U36 in patients with head and neck cancer (24-26). PET imaging would have advantages over SPECT in terms of spatial resolution, signal to noise ratios and quantification.

In conclusion, ^{111}In-mapatumumab tumor uptake in this group of patients was variable and the value of tumor imaging in upfront patient selection for mapatumumab-based treatment needs further evaluation.

Acknowledgments: The authors would like to thank Dr. Robbert Cool for the BIAcore analysis and Dr. Hans Proost and Astrid Horsels for their assistance during the pharmacokinetic calculations.

Grants: Human Genome Sciences, Inc. provided unlabelled mapatumumab. RUG 2005-3365 from The Dutch Cancer Foundation.

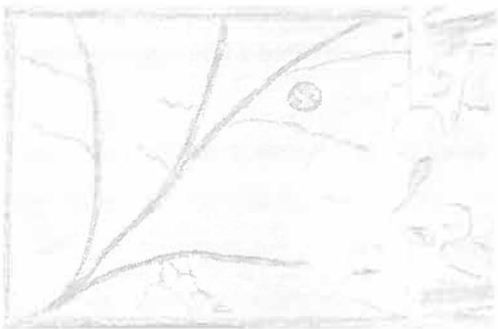
Disclosure of Potential Conflicts of Interest: Norma Lynn Fox and Jerry Klein are employees of Human Genome Sciences and own stock/stock options in Human Genome Sciences.

References

1. Hotte SJ, Hirte HW, Chen EX, Siu LL, Le LH, Corey A, et al. A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies. Clin Cancer Res 2008;14:3450-5.
2. Tolcher AW, Mita M, Meropol NJ, von Mehren M, Patnaik A, Padavic K, et al. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. J Clin Oncol 2007;25:1390-5.
3. Greco FA, Bonomi P, Crawford J, Kelly K, Oh Y, Halpern W, et al. Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer. Lung Cancer 2008;61:82-90.
4. Trarbach T, Moehler M, Heinemann V, Köhne CH, Przyborek M, Schultz C, et al. Phase II trial of mapatumumab, a fully human agonistic monoclonal antibody that targets and activates the tumour necrosis factor apoptosis-inducing ligand receptor-1 (TRAIL-R1), in patients with refractory colorectal cancer. Br J Cancer 2010;102:506-12.
5. Younes A, Vose JM, Zelenetz AD, Smith MR, Burris HA, Ansell SM, et al. A Phase 1b/2 trial of mapatumumab in patients with relapsed/refractory non-Hodgkin's lymphoma. Br J Cancer 2010;103:1783-7.
6. Mom CH, Verweij J, Oldenhuis CN, Gietema JA, Fox NL, Miceli R, et al. Mapatumumab, a fully human agonistic monoclonal antibody that targets TRAIL-R1, in combination with gemcitabine and cisplatin: a phase I study. Clin Cancer Res 2009;15:5584-90.
7. Gong J, Yang D, Kohanim S, Humphreys R, Broemeling L, Kurzrock R. Novel in vivo imaging shows up-regulation of death receptors by paclitaxel and correlates with enhanced antitumor effects of receptor agonist antibodies. Mol Cancer Ther 2006; 5:2991-3000.
8. Duiker EW, Dijkers EC, Lambers Heerspink H, de Jong S, van der Zee AG, Jager PL, et al. Development of radioiodinated rhTRAIL and radiolabelled agonistic TRAIL receptor antibodies for clinical imaging studies. Accepted for publication by the British Journal of Pharmacology. DOI: 10.1111/j.1476-5381.2011.01718.x
9. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205-16.
10. Dijkers EC, Kosterink JG, Rademaker AP, Perk LR, van Dongen GA, Bart J, et al. Development and characterization of clinical-grade 89Zr-trastuzumab for HER2/neu immunoPET imaging. J Nucl Med 2009;50:974-81.
11. Buijs WC, Massuger LF, Claessens RA, Kennemans P, Corstens FH. Dosimetric evaluation of immunoscintigraphy using indium-111-labeled monoclonal antibody fragments in patients with ovarian cancer. J Nucl Med 1992;33:1113-20.
12. Buijs WC, Siegel JA, Boerman OC, Corstens FH. Absolute organ activity estimated by five different methods of background correction. J Nucl Med 1998;39:2167-72.
13. Visser E, Postema E, Boerman O, Visschers J, Oyen W, Corstens F. Software package for integrated data processing for internal dose assessment in nuclear medicine (SPRIND). Eur J Nucl Med Mol Imaging 2007;34:413-21.
14. Stabin MG, Sparks RB. Olinda-PC-based software for biokinetic analysis and internal dose calculations in nuclear medicine. J Nucl Med 2003;44:103P.
15. Loening AM, Gambhir SS. AMIDE: a free software tool for multimodality medical image analysis. Mol Imaging 2003;2:131-7.
16. Nagengast WB, Lub-de Hooge MN, van Straten EM, Kruijf S, Brouwers AH, den Dunnen WF, et al. VEGF-SPECT with (111)In-bevacizumab in stage III/IV melanoma patients. Eur J Cancer 2011;47:1595-602.
17. Koornstra JJ, Kleibeuker JH, van Geelen CM, Rijcken FE, Hollema H, de Vries EG, et al. Expression of TRAIL (TNF-related apoptosis-inducing ligand) and its receptors in normal colonic mucosa, adenomas, and carcinomas. J Pathol 2003;200:327-35.

18. Dijkers EC, de Vries EG, Kosterink JG, Brouwers AH, Lub-de Hooge MN. Immunoscintigraphy as potential tool in the clinical evaluation of HER2/neu targeted therapy. Curr Pharm Des 2008;14:3348-62.
19. Zhang Y, Yoshida T, Zhang B. TRAIL induces endocytosis of its death receptors in MDA-MB-231 breast cancer cells. Cancer Biol Ther 2009;8:917-22.
20. McCarthy MM, DiVito KA, Sznol M, Kovacs D, Halaban R, Berger AJ, et al. Expression of tumor necrosis factor-related apoptosis-inducing ligand receptors 1 and 2 in melanoma. Clin Cancer Res 2006;12:3856-63.
21. Zhuang L, Lee CS, Scolyer RA, McCarthy, Zhang XD, Thompson JF, et al. Progression in melanoma is associated with decreased expression of death receptors for tumor necrosis factor-related apoptosis-inducing ligand. Hum Pathol 2006;37:1286-94.
22. Fisher RE, Siegel BA, Edell SL, Oyesiku NM, Morgenstern DE, Messmann RA, et al. Exploratory study of 99mTc-EC20 imaging for identifying patients with folate receptor-positive solid tumors. J Nucl Med 2008;49:899-906.
23. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001;344:783-92.
24. Dijkers EC, Oude Munnink TH, Kosterink JG, Brouwers AH, Jager PL, de Jong JR, et al. Biodistribution of 89Zr-trastuzumab and PET imaging of HER2-positive lesions in patients with metastatic breast cancer. Clin Pharmacol Ther 2010;87:586-92.
25. Oude Munnink TH, Korte MA, Nagengast WB, Timmer-Bosscha H, Schröder CP, de Jong JR, et al. (89)Zr-trastuzumab PET visualises HER2 downregulation by the HSP90 inhibitor NVP-AUY922 in a human tumour xenograft. Eur J Cancer 2010;46:678-84.
26. Börjesson PK, Jauw YW, Boellaard R, de Bree R, Comans EF, Roos JC, et al. Performance of immuno-positron emission tomography with zirconium-89-labeled chimeric monoclonal antibody U36 in the detection of lymph node metastases in head and neck cancer patients. Clin Cancer Res 2006;12:2133-40.

CHAPTER 8



Single nucleotide polymorphism A683C in TRAIL-receptor 1 and disease outcome after mapatumumab: an exploratory study

CNAM Oldenhuis[1], S Fulda[2], S Sleijfer[3], HM Boezen[4], D Mertens[5], EGE de Vries[1], J Verweij[3], JA Gietema[1], S de Jong[1].

Departments of [1]Medical Oncology and [4]Epidemiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; [2]Institute for Experimental Cancer Research in Pediatrics, Goethe-University Frankfurt/Main, Germany; [3]Department of Medical Oncology, Erasmus University Medical Center Rotterdam, Daniel den Hoed Cancer Center, Rotterdam, The Netherlands; [5]Department of Internal Medicine III, Ulm University, Ulm, Germany.

Submitted.

Abstract

Background: The extrinsic apoptotic pathway is activated in tumor cells after ligand binding to the death receptors TRAIL-R1 and/or TRAIL-R2. Mutations in these death receptors might play a role in insufficient apoptosis induction. Recently, the single nucleotide polymorphism (SNP) A683C in the TRAIL-R1 gene *TNFRSF10A* was identified. Cancer cell lines with the 683C variant expressing the TRAIL-R1 Glu228Ala variant are less sensitive to rhTRAIL than the wild type carriers. We investigated this SNP in genomic DNA samples of patients treated with a regimen that contained a TRAIL-R1 targeting antibody, mapatumumab.

Methods: DNA was isolated from plasma samples of patients with advanced solid tumors. Patients were treated with standard doses of gemcitabine and cisplatin, and escalating doses of mapatumumab and analyzed for SNP A683C (rs20576) in *TNFRSF10A*. Association of the genotype data with clinical phenotypes including progression-free survival (PFS) and treatment related toxicity was studied in order to determine a possible predictive value of this SNP for response to a mapatumumab-containing treatment.

Results: TRAIL-R1 polymorphism A683C was analyzed in 44 patients, 33 expressed the 683A variant, while 9 patients displayed 683A/C heterozygosity. Two patients were homozygous for the 683C variant. Patients heterozygous or homozygous for the 683C variant were analyzed as one group. PFS between 683A and 683C carriers did not differ. No difference was present between both groups with respect to baseline characteristics and treatment related hematological toxicity.

Conclusion: In this first study exploring the effect of SNP *TNFRSF10A* 683C on mapatumumab-containing treatment we found no difference in PFS or treatment related hematological toxicity. Given the small, exploratory nature of our study, an effect on disease outcome in the subgroup of patients carrying the *TNFRSF10A* 683C variant can not be ruled out. Larger prospective studies are therefore needed.

Introduction

Induction of apoptosis is an important factor in anticancer therapy. Chemotherapy and radiotherapy both induce apoptosis by activating the intrinsic, p53-dependent apoptotic pathway. In the past 15 years, research has focused on unraveling another apoptosis pathway, known as the extrinsic pathway. The extrinsic pathway can be activated upon binding of the naturally occurring tumor necrosis factor-related apoptosis inducing ligand (TRAIL) to the death receptors TRAIL-receptor 1 (TRAIL-R1; DR4) and TRAIL-R2 (DR5) (1).

TRAIL-R targeting agents are currently in clinical development in a recombinant soluble human form (rhTRAIL/dulanermin) and as agonistic monoclonal antibodies specifically targeting TRAIL-R1 or TRAIL-R2. Mapatumumab is a fully human monoclonal antibody against the TRAIL-R1 and induces cell death in tumor cells in vitro and in preclinical vivo models, while sparing normal cells. Phase I and II clinical studies showed that mapatumumab can be safely administered both as single agent and in combination with chemotherapy (2-6). The precise role of mapatumumab as anticancer agent remains of course to be established in randomized phase 3 trials. However, as holds true for all anti-tumor regimens, one can expect that only a subset of cancer patients will benefit from this targeted therapy underlining the need for biomarkers allowing the selection of patients who are likely to benefit from mapatumumab-containing approaches.

The gene encoding for the death receptor TRAIL-R1, *TNFRSF10A*, is located on chromosome 8p21. Wolf et al. studied a single nucleotide polymorphism (SNP) in this gene (7). The A -> C nucleotide exchange on position 683 of the *TNFRSF10A* sequence resulted in the amino acid substitution Glu228Ala in the cysteine-rich interaction domain 3 (CRD3) of the TRAIL-R1. *TNFRSF10A* 683C heterozygosity was more common in germline DNA of head and neck squamous cell carcinoma, chronic lymphocytic leukemia (CLL), bladder cancer, and prostate cancer patients compared to healthy controls. Moreover, CLL and prostate cancer cell lines carrying the Glu228Ala variant were less sensitive to rhTRAIL. Apoptosis signaling via TRAIL-R1 could be restored using

mutated TRAIL-like peptides, inducing caspase-8 activation and subsequently apoptosis (8).

The higher incidence of various cancer types and the in vitro resistance to rhTRAIL in cells carrying the 683C variant is possibly caused by an insufficient complex formation due to reduced rhTRAIL binding and therefore less apoptosis (7,8). Whether cancer patients with the germline SNP A683C in *TNFRSF10A* are less sensitive to TRAIL-R1 targeting therapy such as rhTRAIL and agonistic TRAIL-R1 antibody is unknown. To our knowledge this is the first study evaluating the TRAIL-R1 polymorphism A683C in patients treated with TRAIL-R1 targeting therapy. For this purpose we performed a retrospective analysis of the TRAIL-R1 polymorphism A683C in relation to disease outcome and toxicity in patients treated with a mapatumumab-containing regimen in prospective clinical trials.

Material and methods

Study design and patients

Patients were treated as reported earlier in the context of a phase 1 study and an imaging study (NTR2103) (6,9). In total 61 patients received standard doses of gemcitabine and cisplatin, and escalating doses of mapatumumab. Briefly, patients with advanced solid tumors received the combination treatment in 21-day cycles. Evaluation of response according to RECIST 1.0 was performed every second cycle in the phase 1 study and after every third cycle in the imaging study (10). After 6 cycles of chemotherapy and in absence of disease progression, patients could continue therapy with mapatumumab alone. Toxicity was carefully monitored using the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI-CTCAE) version 3.0.

The Ethics Committee approved the study and all patients provided written informed consent for participation in the phase 1 study or imaging study.

DNA collection, amplification and genotyping

Germline DNA was isolated from baseline plasma samples using the QIAamp DNA Mini Kit Spin Protocol for plasma samples (Qiagen GmbH) according to the protocol provided by the manufacturer, as has been previously described (11).

Genomic DNA Fragments for *TNFRSF10A* were amplified using Eurotaq Polymerase (BioCat) with primers (Biospring) as published previously (7). The nucleotide sequence of *TNFRSF10A* was determined by cycle sequencing with Big Dye terminator chemistry (Applied Biosystems) followed by electrophoresis on a Perkin Elmer ABI-377 automated sequencer. Variants of 683A/C were clearly visible as a double peak in the nucleotide sequence.

Correlation study

Patient characteristics used for assessing differences between *TNFRSF10A* 683A wild type versus *TNFRSF10A* 683C variant SNP carriers included gender, age, tumor type, dosing cohort of mapatumumab, number of cycles chemotherapy and mapatumumab administered, total cumulative doses mapatumumab, gemcitabine and cisplatin administered, best observed tumor response according to RECIST 1.0, percent change in the size of total tumor lesions, occurrence of grade 3 and/or grade 4 thrombocytopenia, occurrence of grade 3 and/or grade 4 lymphocytopenia and progression-free survival (PFS). PFS was defined as the time elapsing from study inclusion until tumor progression or death, whatever came first.

Statistics

Homozygous and heterozygous variants were combined. Wild type and variant SNP carriers were compared for patient characteristics using the Chi-square test for categoric variables and the Mann-Whitney *U* test for continuous data. The association between PFS with genotypes was estimated using the Kaplan-Meier method and tested formally using the log-rank test. $P < .05$ was considered significant and reported values are two sided. SPSS v16.0 (SPSS Inc.) was used for all statistical analyses.

Results

Patient characteristics are summarized in Table 1. Good quality DNA could be obtained of 44 patients. Thirty-three patients (75%) expressed the *TNFRSF10A* 683A wild type and 9 patients were heterozygous for the *TNFRSF10A* variant 683C. Two patients were homozygous for the *TNFRSF10A* 683C variant. Patient characteristics did not differ between *TNFRSF10A* 683A wild type carriers and *TNFRSF10A* 683C variant carriers. No statistical difference was present between both groups with respect to PFS. Age, gender, best observed tumor response, tumor type, number of chemotherapy cycles administered, number of additional cycles monotherapy mapatumumab, and the dosing cohort mapatumumab did not differ. The occurrence of grade 3 and grade 4 lymphocytopenia and thrombocytopenia was equal between both groups. No differences in cumulative doses gemcitabine, cisplatin or mapatumumab were found.

Survival and polymorphism A683C.

The Kaplan-Meier curve for PFS is shown in Figure 1. Log-rank testing shows no difference in PFS between genotypes (P = .451).

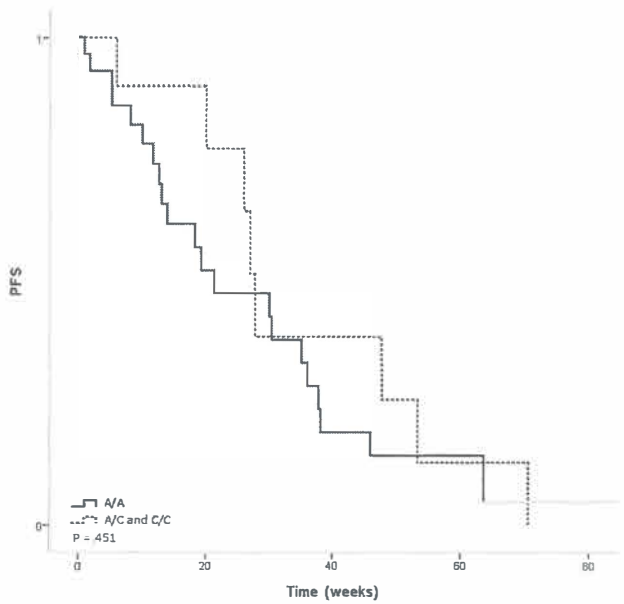


Figure 1. Kaplan-Meier estimate of progression-free survival (PFS) according to *TNFRSF10A* genotype. A/A, wild type; A/C heterozygous variant; C/C homozygous variant.

Table 1. Patient characteristics.

	No. of patients (N = 44)		
	A/A (N = 33)	**A/C and C/C (N = 11)**	***P****
Median age, years (range)	53 (34-72)	54 (42-69)	.728
Gender (male/female)	21/12	8/3	.582
Cohort mapatumumab (mg/kg)			
1	1	1	.789
3	5	2	
10	7	1	
20	11	3	
30	9	4	
Tumor type			.094
Pancreatic cancer	12	6	
(A)CUP	8	0	
NSCLC	4	0	
Cholangiocarcinoma	1	2	
Bladder cancer	1	0	
Head and neck cancer	0	1	
Other	7	2	
Median no. of cycles chemotherapy (range)	4 (1-6)	6 (2-6)	.117
Median no. of cycles monotherapy mapatumumab (range)	0 (0-55)	0 (0-12)	.957
Median duration of progression-free survival, weeks (range)	16.9 (0.9-180.0)	26.5 (6.1-70.8)	.145
Median change in sum of tumor diameter, percentage (range)	-23 (-100-22.4)	-32.7 (-100-7.0)	.578
Best observed response			
NE	4	1	.608
SD	17	6	
PR	7	3	
PD	5	1	
Grade 3 and/or 4 lymphopenia	20	8	.469
Grade 3 and/or 4 thrombocytopenia	15	6	.601
Median cumulative mapatumumab dose, mg (range)	3430 (289-58320)	7740 (350-23882)	.354
Median cumulative gemcitabine dose, mg (range)	14935 (1950-31200)	20400 (4475-30000)	.158
Median cumulative cisplatin dose, mg (range)	510 (130-1020)	687 (285-960)	.105

*Mann-Whitney *U* for continuous data and Chi-square for categorical data.

Abbreviations: (A)CUP, (adeno)carcinoma of unknown primary; NSCLC, non-small cell lung carcinoma; NE, not evaluable; SD, stable disease; PR, partial response; PD, progressive disease.

Discussion

We studied the association of the A -> C nucleotide exchange on position 683 of *TNFRSF10A*, resulting in the amino acid substitution Glu228Ala in the CRD3 of TRAIL-R1, with disease outcome and toxicity in patients with advanced solid tumors treated with gemcitabine, cisplatin and the TRAIL-R1 antibody mapatumumab. PFS did not differ between both groups. *TNFRSF10A* 683A and 683C carriers did not differ with respect to baseline patient characteristics, best observed tumor response and hematological toxicity.

With the emerging development of tumor biology driven drugs there is a growing need for easy obtainable predictive biomarkers. These biomarkers would make it possible to select patients upfront, thereby preventing non-responders from unnecessary potential toxicity and time loss (12). Previous studies have demonstrated that the presence of the *TNFRSF10A* 683C variant is also associated with an increased risk for several cancer types (7,8,14-18). As a consequence of the presence of the *TNFRSF10A* 683C variant, the negatively charged amino acid glutamate on position 228 of TRAIL-R1 is replaced by the uncharged amino acid alanine, which affects the TRAIL binding domain of TRAIL-R1 and therefore likely results in insufficient complex formation (7,8,14-18). After TRAIL binding, sufficient trimerization of the death receptors TRAIL-R1 or TRAIL-R2 is needed in order to form the death inducing signaling complexes (DISCs), which then result in activation of initiator and downstream caspases (13). It was therefore expected that the presence of the *TNFRSF10A* 683C variant would affect the severity of side effects, or, likely negatively, affect responses of tumors to mapatumumab. However, no effect on PFS or treatment toxicity was observed. The equal disease outcome between *TNFRSF10A* 683A/C and *TNFRSF10A* 683A carriers may point at a differential effect of the Glu228Ala substitution on monoclonal antibody binding and rhTRAIL binding to TRAIL-R1 and thus on apoptosis induction. The crystal structure of TRAIL-R1 interacting with rhTRAIL or mapatumumab is not available yet. The interaction sites of rhTRAIL in complex with TRAIL-R2 are supposed to be similar to those of rhTRAIL in complex with TRAIL-R1. Therefore, the crystal structure of TRAIL-R2 in complex with either rhTRAIL or an agonistic TRAIL-R2 antibody

(PRO95780) may help to gain further insight into antibody and TRAIL binding to TRAIL-R1 (19-21). Significant overlap in TRAIL-R2 binding modes was found for rhTRAIL and the TRAIL-R2 antibody. Both rhTRAIL and the TRAIL-R2 antibody bind to CRD3 of TRAIL-R2, which shows conformational diversity and is supposed to be of major importance for TRAIL-R2 activation (21). Glu228 of TRAIL-R1 is located within a stretch of amino acids in the C-terminal part of CRD3 close to the transmembrane domain that is identical to a stretch of 15 amino acids of TRAIL-R2. No direct interaction between the TRAIL-R2 antibody and this part of TRAIL-R2 CDR3 was observed. These data suggest that the binding of mapatumumab in contrast to rhTRAIL will not be affected by the Glu228Ala substitution in the TRAIL-R1 variant. In line with this hypothesis, our clinical data show no difference between patient characteristics like PFS or treatment toxicity after mapatumumab-containing therapy between patients expressing wild type TRAIL-R1 and patients expressing the TRAIL-R1 Glu228Ala variant.

Given the small, exploratory nature of our study, an effect on disease outcome in the subgroup of patients carrying the TRAIL-R1 Glu228Ala variant can not be ruled out. Larger prospective studies are needed to definite exclude an effect of the TRAIL-R1 Glu228Ala variant on activation of the extrinsic pathway in tumors.

References

1. Oldenhuis CN, Stegehuis JH, Walenkamp AM, de Jong S, de Vries EG. Targeting TRAIL death receptors. Curr Opin Pharmacol 2008;8:433-9.
2. Tolcher AW, Mita M, Meropol NJ, von Mehren M, Patnaik A, Padavic K, et al. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. J Clin Oncol 2007;25:1390-5.
3. Hotte SJ, Hirte HW, Chen EX, Siu LL, Le LH, Corey A, et al. A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies. Clin Cancer Res 2008;14:3450-5.
4. Greco FA, Bonomi P, Crawford J, Kelly K, Oh Y, Halpern W, et al. Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer. Lung Cancer 2008;61:82-90.
5. Trarbach T, Moehler M, Heinemann V, Köhne CH, Przyborek M, Schulz C, et al. Phase II trial of mapatumumab, a fully human agonistic monoclonal antibody that targets and activates the tumour necrosis factor apoptosis-inducing ligand receptor-1 (TRAIL-R1), in patients with refractory colorectal cancer. Br J Cancer 2010;102:506-12.
6. Mom CH, Verweij J, Oldenhuis CN, Gietema JA, Fox NL, Miceli R, et al. Mapatumumab, a fully human agonistic monoclonal antibody that targets TRAIL-R1, in combination with gemcitabine and cisplatin: a phase I study. Clin Cancer Res 2009;15:5584-90.
7. Wolf S, Mertens D, Pscherer A, Schroeter P, Winkler D, Gröne HJ, et al. Ala228 variant of trail receptor 1 affecting the ligand binding site is associated with chronic lymphocytic leukemia, mantle cell lymphoma, prostate cancer, head and neck squamous cell carcinoma and bladder cancer. Int J Cancer 2006;118:1831-5.
8. http://www.faqs.org/patents/app/20090203587
9. Oldenhuis CN, Dijkers EC, Duiker EW, Fox NL, Klein JL, Gietema JA, et al. Development of radiolabeled mapatumumab and imaging in solid tumor patients who are treated with gemcitabine, cisplatin, and mapatumumab. J Clin Oncol, 2009;27(suppl; abstr e14521).
10. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205-16.
11. De Haas EC, Zwart N, Meijer C, Nuver J, Boezen HM, Suurmeijer AJ, et al. Variation in bleomycin hydrolase gene is associated with reduced survival after chemotherapy for testicular germ cell cancer. J Clin Oncol 2008;26:1817-23.
12. Oldenhuis CN, Oosting SF, Gietema JA, de Vries EG. Prognostic versus predictive value of biomarkers in oncology. Eur J Cancer 2008;44:946-53.
13. Pennarun B, Meijer A, de Vries EG, Kleibeuker JH, Kruyt F, de Jong S. Playing the DISC: Turning on TRAIL death receptor-mediated apoptosis in cancer. Biochym Biophys Acta 2010;1805:123-40.
14. Chen B, Liu S, Wang XL, Wei X, Yan L, Zhao WH, et al. TRAIL-R1 polymorphisms and cancer susceptibility: An evidence-based meta-analysis. Eur J Cancer 2009;45:2598-605.
15. Dechant MJ, Fellenberg J, Scheuerpflug CG, Ewerbeck V, Debatin KM. Mutation analysis of the apoptotic "death-receptors" and the adaptors TRADD and FADD/MORT-1 in osteosarcoma tumor samples and osteosarcoma cell lines. Int J Cancer 2004;109:661-7.
16. Langsenlehner T, Langsenlehner U, Renner W, Kapp KS, Krippl P, Hofmann G, et al. The Glu228Ala polymorphism in the ligand binding domain of death receptor 4 is associated with increased risk for prostate cancer metastases. Prostate 2008;68:264-8.
17. Frank B, Shanmugam KS, Beckmann L, Hemminki K, Brenner H, Hoffmeister M, et al. Death receptor 4 variants and colorectal cancer risk. Cancer Epidemiol Biomarkers Prev 2006;15:2002-5.
18. Frank B, Hemminki K, Shanmugam KS, Meindl A, Klaes R, Schmutzler RK, et al. Association of death receptor 4 haplotype 626C-683C with an increased breast cancer risk. Carcinogenesis 2005;26:1975-7.
19. Hymowitz SG, Christinger HW, Fuh G, Ultsch M, O'Connell M, Kelley RF, et al. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. Mol Cell 1999;4:563-71.

20. Li B, Russell SJ, Compaan DM, Totpal K, Marsters SA, Ashkenazi A, et al. Activation of the proapoptotic death receptor DR5 by oligomeric peptide and antibody agonists. J Mol Biol 2006;361:522-36.

21. Adams C, Totpal K, Lawrence D, Marsters S, Pitti R, Yee S, et al. Structural and functional analysis of the interaction between the agonistic monoclonal antibody apomab and the proapoptotic receptor DR5. Cell Death Differ 2008;15:751-61.

CHAPTER 9

Summary, general discussion and future perspectives

In the past four decades, the mainstay of anticancer treatment has been surgery, radiotherapy, chemotherapy and hormonal systemic treatment. Despite improvement in these treatment modalities, still half of the cancer patients die as a consequence of metastatic disease. This is partly due to the development of resistance to the administered systemic treatment. Currently, much information is becoming available from tumor cell biology research, opening opportunities for the development of specifically tumor targeting agents.

Chemotherapy-resistant tumor cells are characterized by an intrinsic or acquired incapability for going into apoptosis as a consequence of defects in the intrinsic apoptotic pathway. A strategy to overcome this resistance might be targeting the extrinsic apoptotic pathway. This pathway is activated by the endogenous occurring Tumor Necrosis Factor (TNF) Related Apoptosis-Inducing Ligand (TRAIL, or Apo2L). TRAIL binds to five different receptors, but induces apoptosis only after binding to its two death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5). The other three receptors, TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and the soluble osteoprotegerin (OPG), act as decoys. The discovery that TRAIL only induces apoptosis in several tumor cells and not in normal cells makes it a potentially attractive anticancer drug. Numerous preclinical studies showed synergy when combining these TRAIL-receptor targeting agents with chemotherapy. In addition, many chemotherapy-resistant cell lines as well as xenograft models could be sensitized by the addition of recombinant human (rh) TRAIL or one of the agonistic death receptor antibodies. RhTRAIL, targeting all TRAIL-Rs, and monoclonal antibodies that target either TRAIL-R1 or TRAIL-R2 were entered into clinical trials and appeared to be safe as single agents in phase 1 and phase 2 trials.

Another approach to exploit a targeted therapy, is to treat malignancies by blocking angiogenesis, the formation of new blood vessels. Tumors require an adequate vascularization to obtain oxygen and nutrients, thereby enabling tumor growth and the development of metastases. An important proangiogenic protein is Vascular Endothelial Growth Factor (VEGF), known to be overexpressed by many tumor types. The binding of VEGF to the VEGF-receptors (VEGFR) can be prevented by drugs

blocking VEGF directly or by binding to the tyrosine kinase domain of the involved receptors.

TRAIL-R targeting agents and angiogenesis inhibitors are examples of targeted therapies, i.e. drugs acting against a specific tumor cell characteristic. Anticancer drugs, and targeted therapies, may be beneficial for those patients, where the targeted mechanism is relevant for tumor growth and progression. Upfront patient selection before therapy is initiated or early after start of treatment would clearly help avoid useless treatment for non-responders and avoid unnecessary toxicity.

The objective of this thesis was to study the safety, tolerability and pharmacokinetics of novel targeted agents and to concurrently develop predictive biomarkers for patient selection.

In **chapter 2**, an overview of the TRAIL pathway is presented. The literature is summarized with respect to the physiological and pathophysiological role of TRAIL and the potential exploitation of this pathway for drug treatment. Endogenous TRAIL has anti-inflammatory capacities as well as a role in autoimmunity. Moreover, recent studies showed antitumor surveillance is another property of TRAIL and its receptors. TRAIL-R deficiency promotes primary tumor development and results in faster formation of metastases in mice. On the other hand, in rhTRAIL resistant preclinical models, prosurvival signaling was observed after rhTRAIL-induced death receptor activation resulting in reduced apoptosis and an increase in proliferation and subsequently tumor growth.

RhTRAIL and agonistic antibodies targeting specifically TRAIL-R1 or TRAIL-R2 are in early clinical development and showed until now limited toxicity. Ongoing studies focus especially on combination of these agents with other targeted therapies or cytotoxic therapies. We summarized current knowledge on these agents and highlighted their potential role in the intrinsically chemotherapy-resistant glioblastomas. In addition, we discussed in more detail the mechanisms to sensitize tumors cells to rhTRAIL by combination with the proteasome inhibitor bortezomib.

In the phase 1 study described in **chapter 3**, we evaluated the safety, tolerability, pharmacokinetics, and antitumor activity of the agonistic TRAIL-R1 targeting monoclonal antibody mapatumumab in combination with standard combination regimen with gemcitabine and cisplatin. Patients with advanced solid tumors received gemcitabine 1250 mg/m^2 intravenously (iv) on days 1 and 8 and cisplatin 80 mg/m^2 iv on day 1 of each 21-day cycle. Escalating mapatumumab doses (iv) were administered every 21 days. Toxicity was closely monitored and pharmacokinetic analysis of plasma mapatumumab, gemcitabine, its metabolite 2-difluoro-2-deoxyuridine, and unbound and total platinum was performed. TRAIL-R1 expression on tumor material was determined by immunohistochemistry.
In this study, 49 patients received mapatumumab (1 mg/kg, n = 4; 3 mg/kg, n = 7; 10 mg/kg, n = 12; 20 mg/kg, n = 13; or 30 mg/kg, n = 13). A median of 6 cycles (range, 1-48) was administered. The adverse events most commonly observed reflect the toxicity profile of gemcitabine and cisplatin. Dose-limiting toxicities were seen in 3 of 12 patients at 10 mg/kg, consisting of grade 3 transaminitis, neutropenic fever, and grade 4 thrombocytopenia. At 20 mg/kg, 2 of 12 patients had dose-limiting toxicities, including grade 4 thrombocytopenia and grade 4 fatigue. The maximum tolerated dose was not reached. Pharmacokinetic interactions have not been observed. Twelve patients had a partial response, and 25 patients showed stable disease with a median duration of 6 months. In conclusion, mapatumumab in combination with gemcitabine and cisplatin is safe and well tolerated at doses up to 30 mg/kg. Further studies on this combination are therefore warranted.

Increasingly the critical role of the microenvironment in tumor development, migration and metastasis is acknowledged. A crucial role in the microenvironment is played by angiogenic factors. Tivozanib is an orally available VEGF-R1, VEGF-R2 and VEGF-R3 tyrosine kinase inhibitor that showed potent anticancer activity in vitro and in preclinical in vivo models. Tivozanib appeared to be safe as single agent in advanced solid tumor patients and synergistic effects were observed when combined with chemotherapy in preclinical models.

Encouraged by these data we have performed the, still ongoing, phase 1 study for which prleliminary data are reported in **chapter 4**. In this dose escalation study, tivozanib was combined with fluorouracil, oxaliplatin and leucovorin (FOLFOX6-regimen). Eligible were patients with advanced gastrointestinal malignancies. Tivozanib was administered orally once daily for 21 days in 28-day cycles. Planned daily dose levels of tivozanib were 0.5 mg, 1.0 mg, and 1.5 mg. Standard FOLFOX6 was administered every 14 days. Patients were allowed to continue tivozanib following discontinuation of FOLFOX6 in the absence of disease progression. The safety of administering this combination was observed and pharmacokinetic interactions were studied.

In this ongoing study, 30 patients with a median age of 58 years (range, 40-75 years), were evaluated in the following cohorts of tivozanib: 0.5 mg (n = 9), 1.0 mg (n = 3) and 1.5 mg (n = 18). Dose-limiting toxicities consisted of one episode of reversible grade 4 AST and ALT elevations and 1 reversible grade 3 diarrhea at tivozanib 0.5 mg, no dose limiting toxicities occurred at tivozanib 1.0 mg. At tivozanib 1.5 mg/day dose limiting toxicities consisted of one episode of grade 3 vertigo and one episode of grade 3 epileptic seizure during cycle 2. Other grade 3/4 adverse events across dose levels included hypertension (n = 11) , neutropenia (n = 6) and fatigue (n = 4). There was no indication that drug related adverse events of this combination were more frequent or severe than those observed with FOLFOX6 or tivozanib alone. No PK interactions have been observed. Of the 30 evaluable patients, 1 patient had a complete response, 8 patients received a partial response and 12 experienced stable disease Responses occurred in patients with oesophageal cancer (n = 4), pancreatic cancer (n = 2), colorectal cancer (n = 1), gastric cancer (n = 1) and small bowel cancer (n = 1).

The combination of tivozanib and FOLFOX6 appears feasible and safe, with drug related AEs of the combination that were not more frequent or severe than those known to occur with FOLFOX6 or tivozanib alone The recommended dose for future studies is 1.5 mg/day. Observed clinical activity merits further exploration in gastrointestinal tumors of different origins.

Targeted therapies have in common that they act against a specific feature of tumor cells. Tumor heterogeneity is commonly present, even in patients with the same tumor type, and the target is not always expressed. It is a major challenge to select patients upfront or early during treatment who will benefit from these treatments, while correctly withholding it from the other patients. Identification of, preferably easy obtainable, predictive biomarkers might facilitate this strategy. An attempt to pursue such a strategy is described in the second part of this thesis.

In **chapter 5**, we clarified the confusing terminology "prognostic and predictive biomarkers". A prognostic biomarker provides information about the patient's overall cancer outcome, regardless of therapy, while a predictive biomarker gives information about the effect of a therapeutic intervention. A predictive biomarker can be a target for therapy. We discussed several examples of clinically relevant genes like estrogen receptor (ER), progesterone receptor (PR) and *HER2/neu* in breast cancer and *EGFR1* mutations in non-small cell lung cancer (NSCLC). ER and PR expression are both examples of independent prognostic biomarkers as well as predictive factors for response to endocrine therapy in patients with breast cancer. *HER2/neu* gene amplification results in overexpression of this receptor on the cell membrane and is also of prognostic and predictive value in breast cancer patients. However, in contrast to ER and/or PR overexpression, *HER2/neu* amplification is a negative prognostic factor. The worse prognosis of patients overexpressing HER2/neu is "neutralized" by adding trastuzumab to chemotherapy in the adjuvant setting compared to HER2/neu negative patients. We also discussed that different tumor types can be treated by blocking the same pathway, like for example EGFR inhibition in colorectal cancer and NSCLC. Predictive biomarkers may be shared between tumor types, like the negative predictive value of *K-ras* mutations in colorectal cancer and NSCLC for the effect of EGFR inhibition. However, on the other hand, *EGFR* mutations do predict benefit from anti-EGFR therapy in NSCLC where they do not in colorectal cancer. Identification of predictive biomarkers is important in our effort to develop patient-tailored treatment.

This is an additional argument that biomarker studies should be incorporated into clinical trials.

Approximately 5-10% of the colorectal cancer patients comprise the well-characterized hereditary forms, hereditary nonpolyposis colorectal cancer (HNPCC, or Lynch syndrome) and familial adenomatous polyposis (FAP). Since most of these patients develop colorectal cancer during their life, treatment strategies are sought to prevent tumor development. The non-steroidal anti-inflammatory drug (NSAID) sulindac reduces colorectal cancer risk in FAP patients, where its role in HNPCC is still under debate. The molecular mechanisms underlying these effects are incompletely understood. Many studies suggest an important role for the induction of apoptosis involving the mitochondrial pathway and the death receptor pathway. Alternatively, mechanisms involving the Wingless-int (Wnt) pathway have been suggested, possibly mediated by p21. The Wnt signaling pathway plays an important role in colorectal carcinogenesis. Mutations in the adenomatous polyposis coli (*APC)* or *β-catenin* gene lead to cytoplasmic accumulation and increased translocation to the nucleus of β-catenin. This results in activation of T-cell factor 4 and subsequently activation of a genetic program responsible for adenoma formation. Active Wnt-signaling also reduces p21 levels, allowing cells to proliferate instead of differentiate. Sulindac metabolites showed to decrease β-catenin expression in colon cancer cells. In addition, sulindac induced p21 expression in vitro and in a mouse model.

In the biomarker study described in **chapter 6** we have tried to provide more insight in the chemopreventive mechanisms of sulindac. Biopsies of normal-appearing colonic mucosa obtained during two previously published studies before and after sulindac treatment were analyzed for the presence of apoptotic cells and the expression of TRAIL-R1, TRAIL-R2, p21 and β-catenin by immunohistochemistry. Patients (n = 18) with HNPCC received 150 mg sulindac two times daily for 4 weeks in a placebo-controlled crossover design. Patients (n = 6) with FAP received 150 mg sulindac twice a day for 6 months. Apoptosis was assessed by M30 staining and expression patterns of TRAIL-R1, TRAIL-R2, β-catenin, and p21 were studied immunohistochemically. In

HNPCC patients, apoptotic indices were similar following placebo and sulindac. Also in FAP patients, apoptotic indices were not different after sulindac compared with pretreatment values. Expression of TRAIL-R1 and TRAIL-R2 was observed in all samples with no consistent differences between placebo/baseline and sulindac. Intensity of membranous β-catenin staining was lower in HNPCC samples following sulindac compared with placebo ($p < 0.001$). Similar results were obtained in FAP samples ($p < 0.01$). P21 expression before and after sulindac treatment were similar in both patient groups. We concluded that sulindac inhibits β-catenin expression in normal colorectal epithelium from HNPCC and FAP patients without affecting apoptotic indices and TRAIL-R1, TRAIL-R2, and p21 expression. We, therefore, have no evidence for a role of the TRAIL-R pathway in sulindac-induced chemoprevention. However, our data provide further support for inhibition of the Wnt signaling as a contributing mechanism of chemoprevention by sulindac.

It is unknown whether the TRAIL-R1 targeting monoclonal antibody mapatumumab actually reaches the tumor cells within a patient's tumor to display its anticancer efficacy. Upregulation of TRAIL-R1 after chemotherapy was seen in mice bearing human colorectal cancer tumor xenografts. However the effect of chemotherapy on mapatumumab tumor binding is not clear. In **chapter 7** we described the tumor uptake, pharmacokinetics and biodistribution of ^{111}In-mapatumumab in patients with advanced solid tumors, at the start and during treatment with gemcitabine, cisplatin and mapatumumab. Patients were eligible for the study if they met the inclusion criteria of the preceding phase I study outlined in chapter 3 and were treated accordingly. Patients received 150 MBq ^{111}In-mapatumumab i.v. 1.5 hours following the start of the mapatumumab infusion in cycle 1. The protocol provided the possibility that, if tumor lesions were not clearly visualized after 4 patients, the tracer was administered 7 days before the mapatumumab infusion in cycle 1. On day 2, cycle 3, 150 MBq ^{111}In-mapatumumab was administered 30 minutes before the end of the therapeutic mapatumumab dose. Planar whole body imaging and single-photon emission computed tomography (SPECT) was performed at 0.5, 24, 72, and 144 hours after tracer injection. Venous blood samples were obtained up to 312 hours following

^{111}In-mapatumumab injection. TRAIL-R1 expression was determined immunohistochemically in archival tumor tissue. In 5 of the 12 patients included, 11 out of 18 tumor lesions known by CT were visualized with ^{111}In-mapatumumab SPECT, but a large heterogeneity was seen in ^{111}In-mapatumumab uptake between these 5 patients. Two melanoma patients showed remarkable intense tracer uptake. In 3 patients all lesions were visualized. SPECT results in cycle 3 were comparable to the first SPECT series. Three of the 4 patients having positive ^{111}In-mapatumumab scintigraphy and tumor tissue available showed at least low cytoplasmic TRAIL-R1 expression. Intensity of staining did not correlate with positive ^{111}In-mapatumumab scintigraphy or tumor response to treatment. In conclusion, ^{111}In-mapatumumab tumor uptake in this group of patients was variable and the value of tumor imaging in upfront patient selection for mapatumumab-based treatment needs further evaluation.

The extrinsic apoptotic pathway is activated after ligand binding to TRAIL-R1 and/or TRAIL-R2. Mutations in death receptors or in downstream proteins might play a role in insufficient apoptosis induction. Recently, the single nucleotide polymorphism (SNP) A683C in the *TNFRSF10A* gene encoding TRAIL-R1 was identified. Cancer cell lines expressing the variant appeared to be less sensitive to rhTRAIL compared to the wild type carriers. In **chapter 8** we analyzed the effect of SNP A683C in germline DNA samples of patients treated with gemcitabine, cisplatin and the TRAIL-R1 targeting antibody mapatumumab in the two previously performed studies described in chapter 3 and chapter 7. For this purpose we performed an analysis of the TRAIL-R1 polymorphism A683C in relation to outcome and toxicity. Genomic DNA of 44 patients was successfully analyzed for the *TNFRSF10A* polymorphism A683C. In total, 33 of the 44 patients were carrier of the wild type A683, while 9 patients were heterozygous and 2 patients were homozygous for the variant A683C. The patients with either heterozygous or homozygous variants were combined and analyzed as one group. Progression-free survival between 683A and 683C carriers did not differ. No difference was present between both groups with respect to baseline characteristics and treatment related hematological toxicity. Given the small, exploratory nature of our

study, an effect on disease outcome in the subgroup of patients carrying the *TNFRSF10A* 683C variant can not be ruled out. Larger prospective studies are therefore needed.

In conclusion, we describe in this thesis two phase 1 studies with novel targeted therapies that could be safely combined with chemotherapy. Furthermore, we explored the chemopreventive mechanism of the NSAID sulindac in colorectal cancer and investigated potential predictive biomarkers for mapatumumab-containing treatment. Both the new targeted therapies as well as the biomarkers studied could potentially be implemented in future anticancer treatment strategies.

General discussion and future perspectives

In **chapters 3 and 4**, we have shown that both the TRAIL-R1 targeting monoclonal antibody mapatumumab, as well as the VEGF-R1, 2 and 3 tyrosine kinase inhibitor tivozanib, could be safely combined with chemotherapy. Data on antitumor activity of these compounds needs of course to be established in phase 2 and phase 3 trials. Chemotherapy is still the basis of most systemic anticancer treatment strategies. However, more and more insight in the molecular and oncogenetic characteristics of tumors points towards interesting highly specific targeting agents. Mapatumumab induces apoptosis by ligation of TRAIL-R1 and results in a cascade of caspase activations eventually resulting in apoptosis. More downstream of the apoptotic pathway, apoptosis can be prevented by inhibitor of apoptosis (IAP) proteins, like X-linked IAP (XIAP). IAP inhibitors are currently in early clinical development (1). However, single agent activity seems to be modest. Recently, it was shown that small molecule XIAP inhibitors profoundly enhanced mapatumumab-induced apoptosis in pancreatic carcinoma cell lines (2). A next step in targeting the apoptotic pathway in cancer patients might therefore be combining both apoptosis-inducing treatment modalities.

Tumor cell apoptosis can also be achieved by activating the p53-dependent intrinsic pathway. MDM2 is a negative regulator of p53 and results in proteasomal degradation of p53 after complex formation. Nutlin is a small-molecule that binds MDM2, thereby preventing its interaction with p53 (3). Our group showed enhanced apoptosis induction after combining the MDM2 antagonist Nutlin-3 with TRAIL-R targeting agents (4). Although an interesting concept, the safety results of the currently ongoing single agent phase 1 studies with MDM2 targeting drugs should of course be awaited (NCT01143740, NCT01462175) before combining a MDM2 antagonist with TRAIL-R targeting agents even can be considered.

Mapatumumab and tivozanib target different, but both biological important pathways, each representing a hallmark of cancer as defined by Hanahan and Weinberg (5). As most malignancies are not dependent of one pathway for tumor growth and progression, one can envision that future therapy lies in combinations specifically targeting several pathways, instead of several drugs targeting the same pathway, would be more effective.

Combining new targeted therapies with radiotherapy might be another approach for improving cancer outcome. For example in patients with locally advanced cervical cancer, where the standard treatment consists of radiotherapy and concomitant cisplatin-based chemotherapy. This treatment schedule results in a 5-year overall survival of 66-79%, illustrating that there is ample room for improvement (6). The toxicity profile of radiotherapy and chemotherapy hardly permit dose escalation of current drugs or irradiation. Therefore, the combination with targeted therapies might open a new venue. In cervical cancer cell lines and xenografts, mapatumumab in combination with irradiation resulted in synergistic apoptosis induction (unpublished results, de Jong) (7). We have shown in the phase I trial, described in **chapter 3**, that the addition of mapatumumab to cisplatin did not augment side effects. Currently we are evaluating the combination of mapatumumab with cisplatin and radiotherapy as a first line therapy in patients with advanced cervical cancer in a phase 1b study (NCT01088347).

Reducing cancer mortality can also be achieved by prevention of tumor formation. In **chapter 6** we have shown that inhibition of Wnt signaling might be a contributing mechanism of sulindac-induced chemoprevention in colorectal cancer. No effect was seen on TRAIL-R expression. Recently, our group demonstrated that sulindac effectively sensitized colon adenoma cell lines to rhTRAIL induced apoptosis, also without affecting TRAIL-R expression. Moreover, this effect was only seen in cells with active Wnt signaling (8). Given the favorable toxicity profiles of both NSAIDs and TRAIL-R targeting agents, it would be of interest to combine both drugs in colorectal cancer chemoprevention strategies, especially in the genetic susceptible population.

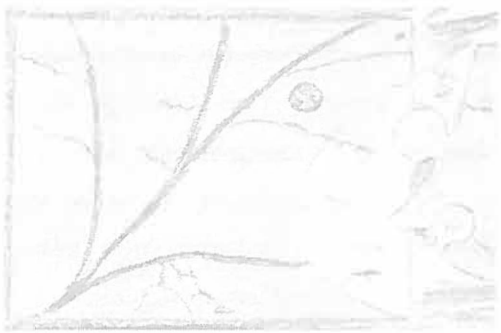
Characteristics of tumors and metastatic lesions will be increasingly leading to the optimal systemic therapy of choice in individual patients. Non-invasive biomarkers that can predict the patients' tumor response before start of treatment are therefore needed. We explored ^{111}In-mapatumumab scintigraphy (**chapter 7**) and TRAIL-R1 683C variant (**chapter 8**) as candidate biomarkers. Both are of potential clinical relevance in selecting patients for mapatumumab-containing treatment, although they need of course validation in larger, prospective trials.

Phase 1 studies in general include patients with advanced cancer for whom no standard treatment is available. In the current era, where new developmental drugs mostly consist of targeted therapies, it would be of interest to select patients in whom the target is available. This will give probably a more reliable safety profile, including specific on-target toxicity, of the drug tested. Next to this, it would be important to put even more effort than is currently done to, prospectively, incorporate analysis of potential biomarkers in early clinical studies. This might not only result in a putative increase in response rates in the selected patient group but also spare the other patients toxic site effects of a non-effective treatment. Furthermore, this might accelerate drug development and will limit the amount of patients needed. Such an approach will eventually lead to personalized treatment based on the molecular profile of an individual patients' tumor and thereby hopefully increase survival in cancer patients.

References

1. Kashkar H. X-linked inhibitor of apoptosis: a chemoresistance factor or a hollow promise. Clin Cancer Res 2010;16:4496-502
2. Stadel D, Mohr A, Ref C, et al. TRAIL-induced apoptosis is preferentially mediated via TRAIL receptor 1 in pancreatic carcinoma cells and profoundly enhanced by XIAP inhibitors. Clin Cancer Res 2010;16:5734-49
3. Brown CJ, Lain S, Verma CS, et al. Awakening guardian angels: drugging the p53 pathway. Nat Rev Cancer 2009;9:862-73
4. Meijer A, Kruyt FA, van der Zee AG, et al. Nutlin-3 preferentially sensitizes wild type p53 expressing cancer cells to DR5-selective rhTRAIL over rhTRAIL. Targeting apoptosis in cancer cells: improving TRAIL death receptor signalling (thesis)
5. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74
6. Vale C, Tierney JF, Stewart LA, et al. Chemoradiotherapy for Cervical Cancer Meta-Analysis Collaboration Reducing uncertainties about the effects of chemoradiotherapy for cervical cancer: a systematic review and meta-analysis of individual patient data from 18 randomized trials. J Clin Oncol 2008;26:5802-12
7. Maduro JH, de Vries EG, Meersma GJ, et al. Targeting pro-apoptotic trail receptors sensitizes HeLa cervical cancer cells to irradiation-induced apoptosis. Int J Radiat Oncol Biol Phys 2008;72:543-52
8. Heijink DM, Jalving M, Oosterhuis D, et al. TNF-related apoptosis-inducing ligand cooperates with NSAIDs via activated Wnt signalling in (pre)malignant colon cells. J Pathol 2011;223:378-89

CHAPTER 10



Samenvatting, algemene discussie en toekomstperspectieven

De afgelopen veertig jaar zijn chirurgie, radiotherapie en systeemtherapie de pijlers van kankerbehandeling geweest. Ondanks gestage uitbreiding van deze therapeutische mogelijkheden overlijdt nog steeds de helft van de kankerpatiënten aan de gevolgen van gemetastaseerde ziekte. Dit is ten dele toe te schrijven aan het ontwikkelen van tumorcelresistentie tegen de toegediende systeemtherapie. Door een verbeterd inzicht in de tumorcelbiologie heeft de ontwikkeling van specifieke, doelgerichte medicijnen op grond van tumoreigenschappen zich de laatste jaren sterk uitgebreid.
Tumorcellen die resistent zijn voor bijvoorbeeld chemotherapie hebben als gevolg van een defecte intrinsieke apoptoseroute een intrinsieke of verworven onvermogen om in apoptose te gaan. Deze resistentie zou potentieel omzeild kunnen worden door activatie van de extrinsieke apoptoseroute. De extrinsieke route wordt geactiveerd door het lichaamseigen Tumor Necrosis Factor (TNF) Related Apoptosis-Inducing Ligand (TRAIL, of Apo2L). TRAIL kan aan vijf verschillende receptoren binden, maar induceert slechts apoptose na binding aan TRAIL-R1 (DR4) of TRAIL-R2 (DR5). De andere drie receptoren, TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) en het oplosbare osteoprotegerine (OPG), fungeren als zogenaamde *decoys*. De ontdekking dat TRAIL alleen apoptose in tumorcellen induceert en niet in de meeste normale cellen, maakt het een potentieel aantrekkelijk antikankermiddel. Vele preklinische studies hebben synergie laten zien indien TRAIL-R1/2 activerende stoffen worden gecombineerd met chemotherapie. Daarnaast worden veel chemotherapieresistente cellijnen en xenograftmodellen opnieuw gevoelig voor het betreffende chemotherapeuticum na toevoeging van recombinant humaan (rh) TRAIL of één van de antilichamen gericht tegen de pro-apoptotische TRAIL-R1/2. RhTRAIL, dat aan alle TRAIL receptoren bindt en monoclonale antilichamen die of TRAIL-R1 of TRAIL-R2 binden, zijn inmiddels in fase 1 en fase 2 studies getest en lijken veilig te zijn.
Een andere benadering om resultaten van oncologische behandelingen mogelijk te verbeteren, is het blokkeren van angiogenese, de vorming van nieuwe bloedvaten. Tumorcellen hebben een adequate bloedvatvoorziening nodig om voorzien te worden van voldoende zuurstof en voedingsstoffen. Hierdoor is de tumor in staat te groeien en te metastaseren. Een belangrijk pro-angiogeen eiwit is Vascular Endothelial Growth

Factor (VEGF), dat door vele tumoren tot overexpressie komt en door die tumoren geseceneerd wordt. De binding van het door de tumor geproduceerde VEGF aan de VEGF-receptoren (VEGFR) op endotheelcellen kan voorkomen worden door medicijnen die VEGF direct blokkeren of de tyrosinekinase-activiteit van de betrokken receptor remmen.

TRAIL-R1/2 activerende middelen en angiogeneseremmers zijn voorbeelden van doelgerichte medicijnen. Deze medicijnen zijn gericht tegen een specifiek kenmerk van een cel. Oncologische behandelingen, inclusief doelgerichte medicijnen, werken vanzelfsprekend alleen als het mechanisme dat geattaqueerd wordt een belangrijke rol speelt in tumorgroei van de betreffende patiënt. Het kunnen selecteren van patiënten vooraf of kort na start van de therapie is van groot belang om onnodige behandeling van en toxiciteit bij patiënten te voorkomen.

Het doel van dit proefschrift was enerzijds het bestuderen van de veiligheid, verdraagbaarheid en farmacokinetiek van nieuwe doelgerichte middelen en anderzijds parallel daaraan het ontwikkelen van predictieve biomarkers voor betere patiëntenselectie.

Na een korte inleiding in **hoofdstuk** 1 wordt in **hoofdstuk 2** een overzicht van de TRAIL/TRAIL-R apoptoseroute gegeven. De literatuur met betrekking tot de fysiologische en pathofysiologische rol van TRAIL wordt samengevat, evenals de potentiële rol voor behandeling met medicatie. Endogeen TRAIL heeft anti-inflammatoire eigenschappen en speelt een rol in autoimmuniteit. Daarnaast werd recent aangetoond dat TRAIL/TRAIL-R antitumorcapaciteiten bezit. TRAIL-R deficiëntie zet aan tot ontwikkeling van primaire tumoren en zorgt in muizen voor snellere vorming van metastasen. Er werd echter ook een pro-survival signaal geobserveerd in rhTRAIL resistente, preklinische modellen. Na activatie van TRAIL-R1/2 door rhTRAIL was er namelijk afname van apoptose en juist toename van proliferatie en tumorgroei. RhTRAIL en antilichamen specifiek gericht tegen TRAIL-R1 of TRAIL-R2 zijn in vroeg-klinische ontwikkeling en laten tot nu toe weinig toxiciteit zien. Lopende studies zijn met name gericht op combinaties van deze nieuwe middelen met andere doelgerichte

medicijnen of chemotherapie. De kennis van rhTRAIL en TRAIL-R1/2 antilichamen is samengevat en hun potentiële rol in de intrinsiek chemotherapieresistente gliobastomen is extra belicht. Daarnaast zijn er verschillende mechanismen bediscussieerd om tumorcellen gevoeliger te maken voor rhTRAIL, zoals bijvoorbeeld door combinatie met de proteasoomremmer bortezemib

In de fase-1 studie beschreven in **hoofdstuk 3** werd de veiligheid, verdraagbaarheid, farmacokinetiek en antitumoreffectiviteit van het tegen TRAIL-R1 gerichte monoclonale antilichaam mapatumumab in combinatie met standaarddoseringen gemcitabine en cisplatine bestudeerd. Patiënten met vergevorderde solide tumoren kregen gemcitabine 1250 mg/m^2 intraveneus (iv) op dag 1 en 8 en cisplatine 80 mg/m^2 iv op dag 1 van elke 3-weekse cyclus. Opklimmende doseringen mapatumumab werden elke 3 weken iv toegediend. Toxiciteit werd nauwgezet bijgehouden en farmacokinetische analyse van plasma concentraties mapatumumab, gemcitabine, de metaboliet 2-difluoro-2-deoxyuridine en ongebonden en totaal platinum vond plaats. Expressie van TRAIL-R1 in de tumor werd bepaald middels immunohistochemie.
49 patiënten kregen mapatumumab (1 mg/kg, n = 4; 3 mg/kg, n = 7; 10 mg/kg, n = 12; 20 mg/kg, n = 13; of 30 mg/kg, n = 13) eenmaal per 3 weken. Mediaan werden 6 kuren (range, 1-48) gegeven. De meest geobserveerde bijwerkingen kwamen overeen met het bijwerkingenprofiel van gemcitabine en cisplatine. Dosislimiterende toxiciteit werd gezien bij 3 van de 12 patiënten op de dosisstap van 10 mg/kg en bestond uit: graad 3 stijging van ASAT en ALAT, neutropene koorts en graad 4 trombocytopenie. Bij 20 mg/kg hadden 2 van de 12 patiënten dosislimiterende toxiciteit bestaande uit graad 4 trombocytopenie en graad 4 vermoeidheid. De maximaal getolereerde dosis werd niet overschreden. Er werden geen farmacokinetische interacties gezien. Twaalf patiënten hadden een partiele tumorrespons en 25 patiënten stabiele ziekte met een mediane duur van 6 maanden.
Concluderend kan gesteld worden dat mapatumumab in combinatie met gemcitabine en cisplatine veilig is en goed verdragen wordt bij doseringen tot en met 30 mg/kg elke

3 weken. Vervolgstudies om deze combinatie verder te evalueren zijn daarom aangewezen.

In toenemende mate wordt het cruciale belang van de micro-omgeving voor ontwikkeling van tumoren, migratie en metastasering duidelijk. Angiogene factoren spelen hierbij een belangrijke rol. Tivozanib is een oraal beschikbare VEGF-R1, -R2 en -R3 tyrosine kinase remmer die zowel in vitro als in preklinische in vivo modellen sterke antitumoractiviteit liet zien. Tivozanib blijkt veilig als monotherapie bij patiënten met gemetastaseerde solide tumoren en laat synergistische antitumor effecten zien in combinatie met chemotherapie in preklinische modellen. Aangemoedigd door deze data, hebben we de in **hoofdstuk 4** gerapporteerde studie opgezet en uitgevoerd. De resultaten gepresenteerd in dit hoofdstuk zijn voorlopig, aangezien de studie nog voortduurt. In deze dosisescalatie studie werd tivozanib gecombineerd met fluorouracil, oxaliplatin en leucovorin (FOLFOX6). Patiënten met vergevorderde gastrointestinale maligniteiten kwamen in aanmerking voor deze studie. Tivozanib werd in tabletvorm gedurende de eerste 21 dagen van elke 28 dagen durende cyclus gegeven. Geplande doseringen van tivozanib waren 0,5 mg, 1,0 mg en 1,5 mg per dag. Standaard kuren FOLFOX6 werden elke 14 dagen gegeven. Patiënten mochten doorgaan met tivozanib na het staken van FOLFOX6, mits er geen sprake was van progressieve ziekte. De veiligheid van deze combinatie werd nauwgezet geobserveerd en farmacokinetische interacties werden bestudeerd.
In deze nog lopende studie werden 30 patiënten met een leeftijd van mediaan 58 jaar (range, 40-75 jaar) geëvalueerd in de volgende cohorten tivozanib: 0,5 mg (n = 9), 1,0 mg (n = 3) en 1,5 mg (n = 18). Dosislimiterende toxiciteit met tivozanib 0,5 mg bestond uit 1 episode reversibele graad 4 ASAT en ALAT stijging en 1 patiënt met reversibele graad 3 diarree. Er was geen sprake van dosislimiterende toxiciteit met tivozanib 1,0 mg. Met tivozanib 1,5 mg bestond de dosislimiterende toxiciteit uit een episode graad 3 duizeligheid en een patiënt met een graad 3 epileptisch insult in de tweede kuur. Andere graad 3/4 bijwerkingen gedurende de studie bestonden tot nu toe met name uit hypertensie (n = 11), neutropenie (n = 6) en vermoeidheid (n = 4). Er waren geen

aanwijzingen dat studiemedicatie-gerelateerde bijwerkingen van deze combinatie meer voorkwamen of ernstiger waren dan de bijwerkingen die werden gezien bij alleen FOLFOX6 of alleen tivozanib. Er werden geen farmacokinetische interacties gezien. Van de 30 evalueerbare patiënten had er 1 een complete tumorrespons, 8 hadden een partiële tumorrespons en 12 patiënten hadden stabiele ziekte. Tumorresponsen werden gezien in patiënten met oesofaguscarcinoom (n = 4), pancreascarcinoom (n = 2), colorectaal carcinoom (n = 1), maagcarcinoom (n = 1) en dunne darm carcinoom (n = 1).
Het combineren van tivozanib met FOLFOX6 lijkt op basis van deze gegevens haalbaar en veilig. De aanbevolen dosis tivozanib voor vervolgstudies is 1,5 mg per dag. De geobserveerde klinische activiteit rechtvaardigt nader onderzoek bij verschillende types gastrointestinale maligniteiten.

Doelgerichte middelen hebben als gemeenschappelijk eigenschap dat ze gericht zijn op een specifiek tumorkenmerk. Heterogeniteit in mate van aanwezigheid van het specifieke tumorkenmerk tussen tumoren komt veel voor, maar kan ook variëren in een patiënt met een bepaalde tumortype. Daarnaast is het specifieke tumorkenmerk ook niet altijd aanwezig. Het is een uitdaging die patiënten te selecteren vooraf aan of vroegtijdig tijdens behandeling, die daadwerkelijk baat hebben bij deze behandelingen en deze niet te geven aan de overige patiënten. Het identificeren van, bij voorkeur makkelijk te verkrijgen, predictieve biomarkers zou dit proces kunnen vergemakkelijken. Een poging hiertoe is beschreven in het tweede deel van dit proefschrift.
In **hoofdstuk 5** hebben we een poging gedaan de verwarrende terminologie van “prognostische en predictieve biomarkers” te verhelderen. Een prognostische biomarker geeft informatie over de prognose van de patiënt, onafhankelijk van een al dan niet gegeven behandeling. Een predictieve biomarker geeft informatie over het effect van een therapeutische interventie. Een predictieve biomarker kan een doelwit van de behandeling zijn. We bespraken verschillende voorbeelden van klinisch relevante genen, zoals de oestrogeenreceptor (ER), progesteronreceptor (PR) en

HER2/neu bij mammacarcinomen en *EGFR1* mutaties bij het niet-kleincellig longcarcinoom (NSCLC). ER en PR expressie zijn beide voorbeelden van onafhankelijke prognostische markers en daarnaast predictieve factoren voor respons op endocriene therapie bij patiënten met mammacarcinoom. *HER2/neu* genamplificatie resulteert in overexpressie van deze receptor op de celmembraan en is ook van zowel predictieve als prognostische waarde bij borstkankerpatiënten. Echter, in tegenstelling tot ER en/of PR overexpressie, is *HER2/neu* amplificatie een negatieve prognostische factor. De slechtere prognose van patiënten met HER2/neu overexpressie vergeleken met HER2/neu negatieve patiënten wordt "geneutraliseerd" door trastuzumab toe te voegen aan chemotherapie in de adjuvante setting. Daarnaast lieten we zien dat verschillende tumortypes behandeld kunnen worden door hetzelfde signaalpad te blokkeren, zoals bijvoorbeeld EGFR remming bij colorectaalcarcinoom en NSCLC. Dezelfde predictieve biomarkers kunnen ook aanwezig zijn in verschillende tumortypes, zoals de negatieve predictieve waarde van *K-ras* mutaties voor het effect van EGFR remming bij het colorectaalcarcinoom en bij NSCLC. Echter, *EGFR* mutaties zijn voorspellend voor respons op anti-EGFR therapie in NSCLC, maar niet bij het colorectaalcarcinoom. Identificatie van predictieve biomarkers is van belang voor onze inspanningen patiëntspecifieke therapie op maat te ontwikkelen. Dit is een extra argument om zoveel mogelijk studies naar biomarkers te incorporeren in klinische studies.

Ongeveer 5-10% van de patiënten met colorectaalcarcinoom hebben een van de twee erfelijke vormen, hereditair nonpolyposis colorectaalcarcinoom (HNPCC) of familiaire adenomateuze polyposis (FAP). Aangezien bijna alle patiënten met deze aandoeningen colorectaalcarcinoom ontwikkelen gedurende hun leven, wordt naarstig gezocht naar strategieën om tumorontwikkeling te voorkomen. Het non-steroidale anti-inflammatoire medicijn (NSAID) sulindac reduceert het risico op colorectaalcarcinoom in FAP patiënten en het effect bij HNPCC patiënten is nog onderwerp van onderzoek. Het moleculaire mechanisme dat ten grondslag ligt aan dit effect is nog niet compleet opgehelderd. Veel studies suggereren een belangrijke rol voor apoptose-inductie via

de intrinsieke of de extrinsieke route. Daarnaast zou de Wingless-int (Wnt) route van invloed zijn, al dan niet gemedieerd door p21. De Wnt route speelt een belangrijke rol in de colorectale carcinogenese. Mutaties in het adenomateuze polyposis coli (APC)-gen of het β-cateninegen leiden tot cytoplasmatische accumulatie en toegenomen translocatie van β-catenine naar de celkern. Dit resulteert in activatie van T-cel factor 4 en aansluitend activatie van een genetisch programma verantwoordelijk voor de vorming van adenomen. Actieve Wnt-signalering resulteert daarnaast in verlaging van de hoeveelheid p21, waardoor cellen kunnen prolifereren in plaats van differentiëren. Sulindac metabolieten zorgen voor verminderde β-catenine-expressie in colorectale carcinoomcellen. Bovenal induceert sulindac p21 expressie in een in vitro muizenmodel. Met deze achtergrond hebben we in de biomarkerstudie, beschreven in **hoofdstuk 6,** geprobeerd meer inzicht te geven in de chemopreventieve mechanismen van sulindac. In biopten van normaal uitziend colonslijmvlies, verkregen bij twee eerder gepubliceerde studies, voor en na behandeling met sulindac, werd immunohistochemisch gekeken naar de aanwezigheid van apoptotische cellen en naar de expressie van TRAIL-R1, TRAIL-R2, p21 en β-catenine. Patiënten (n = 18) met HNPCC kregen twee maal daags 150 mg sulindac gedurende 4 weken in een placebogecontroleerde crossover studie. Patiënten (n = 6) met FAP kregen tweemaal daags 150 mg sulindac gedurende 6 maanden. Apoptotische cellen werden geteld na immunohistochemische aankleuring van de apoptosemarker M30 en expressiepatronen van TRAIL-R1, TRAIL-R2, p21 en β-catenine werden beoordeeld middels immunohistochemie. Zowel bij de HNPCC als de FAP patiënten was er geen verschil in apoptose na behandeling met sulindac. Expressie van TRAIL-R1 en TRAIL-R2 werd gezien in alle coupes, zonder consistent verschil tussen de placebo/sulindac naïeve groep en het aan sulindac blootgestelde weefsel. De intensiteit van membraneuze β-catenine kleuring was lager in de biopten van HNPCC-patiënten behandeld met sulindac vergeleken met de placebogroep ($p < 0.001$). Vergelijkbare resultaten werden verkregen in de FAP groep ($p < 0.01$). P21 expressie voor en na sulindac was vergelijkbaar in beide patiëntengroepen. Sulindac lijkt dus β-catenine expressie te remmen in normaal uitziend colorectaalepitheel van HNPCC en FAP

patiënten zonder een effect op apoptose of op de expressie van TRAIL-R1, TRAIL-R2 en p21. We hebben derhalve geen bewijs kunnen vinden voor een bijdrage van de TRAIL-R route in sulindac-geïnduceerde chemopreventie. Remming van Wnt-signalering lijkt echter wel een rol te spelen in het chemopreventieve mechanisme van sulindac.

Het is niet bekend of het TRAIL-R1 activerende monoklonale antilichaam mapatumumab daadwerkelijk tumorcellen bereikt na toediening aan patiënten. Opregulatie van TRAIL-R1 na chemotherapie werd gezien in humane colorectale tumoren dragende muizen. Het effect van chemotherapie op binding van mapatumumab aan tumoren is echter niet duidelijk. In **hoofdstuk 7** beschreven we de opname in tumoren, farmacokinetiek en biodistributie van ^{111}In-gelabeld mapatumumab bij patiënten met vergevorderde solide tumoren na start en gedurende behandeling met gemcitabine, cisplatine en mapatumumab. Patiënten mochten deelnemen aan deze studie als ze voldeden aan de inclusiecriteria van de hieraan voorafgegane fase 1 studie zoals die beschreven is in hoofdstuk 3. Ook de behandeling was gelijk aan deze studie. Patiënten kregen 150 MBq ^{111}In-mapatumumab iv 1,5 uur na start van het mapatumumabinfuus in de 1^{e} cyclus. Het protocol liet de mogelijkheid open, indien tumorlaesies niet duidelijk zichtbaar waren na inclusie van 4 patiënten, om de tracer 7 dagen voor start van het mapatumumabinfuus in de 1^{e} cyclus toe te dienen. Op de tweede dag van de 3^{e} kuur kregen patiënten, 30 minuten voor het einde van de therapeutische dosis mapatumumab, opnieuw 150 MBq ^{111}In-mapatumumab toegediend. Na 30 minuten en op dag 1, 3 en 6 na injectie van de tracer werden planaire scans van het gehele lichaam en single-photon emission computed tomography (SPECT) scans gemaakt. Veneus bloed werd afgenomen op verschillende tijdstippen tot 312 uur na elke ^{111}In-mapatumumab injectie. TRAIL-R1 expressie in tumorweefsel werd bepaald middels immunohistochemie. In 5 van de 12 patiënten werden 11 van de 18 tumorlaesies, vooraf vastgesteld middels CT, teruggezien op de ^{111}In-mapatumumab SPECT scans. Er was echter een grote mate van heterogeniteit in de tumoropname van ^{111}In-mapatumumab bij deze 5 patiënten. Twee melanoompatiënten lieten opmerkelijk sterke traceropname zien. In 3 van de 5

patiënten werden alle bekende tumorlaesies gevisualiseerd. De SPECT resultaten van de 3e cyclus waren vergelijkbaar met die van de 1e cyclus. Van 4 patiënten met ^{111}In-mapatumumab tumoropname was tumorweefsel beschikbaar, waarin bij 3 patiënten lage cytoplasmatische TRAIL-R1 expressie werd gezien. De intensiteit van aankleuring was niet gecorreleerd met ^{111}In-mapatumumabopname of tumorrespons op de behandeling.
Concluderend was de tumoropname van ^{111}In-mapatumumab in de onderzochte patiëntengroep variabel. De waarde van tumorbeeldvorming bij de selectie van patiënten voor start van behandeling met mapatumumab dient daarom nader geëvalueerd te worden.

De extrinsieke apoptoseroute wordt geactiveerd na binding van een ligand aan TRAIL-R1 en/of TRAIL-R2. Mutaties in deze receptoren of in andere eiwitten lager uit de apoptosecascade spelen mogelijk een rol in insufficiënte apoptose-inductie. Recent werd het mononucleotide polymorfisme (SNP) A683C in het *TNFRSF10A* gen, coderend voor TRAIIL-R1, geïdentificeerd. Kankercellijnen die deze variant tot expressie brengen bleken minder gevoelig voor rhTRAIL vergeleken met de wild types. In **hoofdstuk 8** hebben we het effect van SNP A683C geanalyseerd in kiembaan DNA materiaal van patiënten, behandeld met gemcitabine, cisplatine en mapatumumab, uit de eerder in hoofdstuk 3 en hoofdstuk 7 beschreven studies. Het effect van het TRAIL-R1 polymorfisme A683C in relatie tot toxiciteit en ziektevrije overleving werd hiervoor bestudeerd. Genomisch DNA van 44 patiënten werd succesvol onderzocht op het polymorfisme A683C. Van deze patiënten bleken 33 wild type, 9 patiënten heterozygoot en 2 patiënten homozygoot te zijn voor de variant A683C. De hetero- en homozygote varianten werden gecombineerd en geanalyseerd als een groep. Progressievrije overleving tussen 683A en 683C dragers was niet verschillend. Er bestond geen verschil tussen beide groepen in uitgangskarakteristieken en aan de behandeling gerelateerde toxiciteit. Gezien het kleine, explorerende karakter van deze studie kan een effect op ziekte uitkomst in de subgroep patiënten met SNP 683C niet worden uitgesloten. Daarvoor zijn grotere, prospectieve studies noodzakelijk.

Samenvattend worden in dit proefschrift twee fase 1 studies beschreven met nieuwe, doelgerichte medicijnen die veilig gecombineerd kunnen worden met chemotherapie. Daarnaast hebben we geprobeerd het chemopreventieve mechanisme van het NSAID sulindac bij het colorectaal carcinoom verder te exploreren. Tot slot hebben we potentiële predictieve biomarkers voor mapatumumab-bevattende behandeling onderzocht. Beide nieuwe, doelgerichte middelen en de onderzochte biomarkers zouden potentieel wellicht kunnen worden geïmplementeerd in nieuwe behandelstrategieën tegen kanker.

Algemene discussie en toekomstperspectieven

In **hoofdstuk 3 en 4** hebben we laten zien dat zowel het monoclonale antilichaam mapatumumab, gericht tegen TRAIL-R1 en de VEGF-R1, -R2 en -R3 tyrosinekinaseremmer tivozanib veilig gecombineerd kunnen worden met chemotherapie. Antitumoractiviteit van deze middelen moet uiteraard nog worden vastgesteld in fase 2 en fase 3 studies.

Chemotherapie is nog steeds de basis van de meeste systemische antikankerbehandelingen. Met de toenemende kennis over de moleculaire en oncogenetische kenmerken van tumoren vindt er echter een verschuiving plaats naar doelgerichte middelen, specifiek gericht tegen een bepaald tumorkenmerk. Mapatumumab bindt aan TRAIL-R1 en resulteert, via het activeren van een cascade van caspases, uiteindelijk in apoptose. Lager in deze apoptoseroute, kan celdood nog steeds worden voorkomen door zogenaamde remmers van apoptose (IAP) eiwitten, zoals X-gebonden IAP (XIAP). IAP-remmers worden momenteel getest in fase 1 en fase 2 studies (1). Uit de eerste resultaten blijkt dat het monotherapie-effect maar matig is. Recent werd echter duidelijk dat XIAP-remmers het effect van mapatumumab-geïnduceerde apoptose in pancreascarcinoomcellijnen aanmerkelijk versterken (2). Combinatie van beide apoptose-inducerende middelen zou daarom een logische volgende stap zijn om verder onderzocht te worden in een klinische studie bij kankerpatiënten.

Apoptose van tumorcellen kan ook worden bereikt door activeren van de p53-afhankelijke, intrinsieke apoptoseroute. MDM2 is een negatieve regulator van p53 en resulteert na complexformatie in proteasomale afbraak van p53. Nutlin-3 is een klein molecuul dat bindt aan MDM2, waardoor het de interactie van MDM2 met p53 tegengaat (3). Onze onderzoeksgroep heeft de MDM2 antagonist Nutlin-3 gecombineerd met een TRAIL-R1 agonist, wat een duidelijk versterkt apoptosesignaal tot gevolg had (4). Alhoewel conceptueel interessant, moeten de veiligheidsresultaten van de op dit moment lopende monotherapie fase 1 studies met MDM2 antagonisten (NCT01143740, NCT01462175) natuurlijk worden afgewacht, voordat overwogen kan worden een MDM2 antagonist te combineren met een TRAIL-R1 agonist.

Mapatumumab en tivozanib attaqueren verschillende, maar biologisch belangrijke routes in kankercellen (5). De meeste maligniteiten zijn niet afhankelijk van één route voor tumorgroei en progressie. Voor nieuwe antikankerbehandelingen zullen we dan waarschijnlijk zo rationeel mogelijk middelen moeten blijven combineren die op verschillende routes aangrijpen.

Een andere aanpak voor het verbeteren van tumorbehandeling zou kunnen zijn het combineren van nieuwe, doelgerichte middelen met radiotherapie. Bijvoorbeeld bij patiënten met lokaal uitgebreid cervixcarcinoom, waarbij de standaardbehandeling bestaat uit radiotherapie en tegelijkertijd cisplatine bevattende chemotherapie. Dit behandelschema resulteert in een 5-jaarsoverleving van 66-79%, met nog voldoende ruimte voor verbetering (6). De aanzienlijke toxiciteit van deze gecombineerde behandeling laat geen ruimte meer voor dosisescalatie van de chemotherapeutica of radiotherapie. Derhalve zou toevoegen van doelgerichte medicatie wellicht een optie zijn. In cervixcarcinoomcellijnen en -xenografts, resulteerde mapatumumab in combinatie met irradiatie in synergistische apoptose-inductie (ongepubliceerde resultaten, de Jong) (7). We hebben in de fase 1 studie beschreven in **hoofdstuk 3** al laten zien, dat toevoegen van mapatumumab aan cisplatine niet leidt tot meer bijwerkingen. Op dit moment evalueren we de combinatie van mapatumumab met cisplatine en radiotherapie als eerstelijns behandeling bij patiënten met uitgebreid cervixcarcinoom in een fase 1b studie (NCT01088347).

Verminderen van mortaliteit ten gevolge van kanker kan ook worden bereikt door preventie van de ontwikkeling van maligniteiten. In **hoofdstuk 6** hebben we laten zien dat de Wnt route een van de onderliggende mechanismen zou kunnen zijn waardoor sulindac zorgt voor chemopreventie van colorectaalcarcinoom. Er werd geen effect gezien op TRAIL-R1 expressie. Recent heeft onze onderzoeksgroep laten zien dat sulindac colonadenoomcellen gevoelig maakt voor rhTRAIL-geïnduceerde apoptose. Ook hierbij werd geen effect gezien op TRAIL-R1 expressie. Bovendien werd dit effect alleen waargenomen bij cellen met geactiveerde Wnt-signalering (8). Gezien het milde bijwerkingenprofiel van zowel NSAIDs als TRAIL-R1 agonisten, zou het interessant zijn beide middelen te combineren in chemopreventiestudies voor colorectaalcarcinoom en dan met name in de genetisch belaste populatie.

Kenmerken van zowel tumoren als metastasen zullen in toenemende mate bepalen wat de meest optimale systeemtherapeutische behandeling voor de individuele patiënt zal zijn. Niet-invasieve biomarkers, die een eventuele tumorrespons reeds voor start van deze behandeling kunnen voorspellen, zijn daarvoor zeer welkom. We hebben ^{111}In-mapatumumab scintigrafie (**hoofdstuk 7**) en de TRAIL-R1 683C variant (**hoofdstuk 8**) onderzocht als potentiële biomarkers. Beide zijn van potentieel klinische betekenis, alhoewel ze natuurlijk nog gevalideerd moeten worden in grotere, prospectieve studies.

In fase 1 studies worden patiënten geïncludeerd met vergevorderde tumoren waarvoor geen standaard behandeling (meer) beschikbaar is. Met de huidige ontwikkelingen van met name doelgerichte therapieën, zal men steeds meer patiënten selecteren bij wie het doel voor het medicijn in de tumor ook daadwerkelijk aanwezig is, om zo de kans op het ontwikkelen van nieuwe effectievere (combinatie)therapieën te vergroten. Er is al een toenemend streven ook in fase 1 studies prospectief, potentiële biomarkers te testen. Uiteindelijk zou zo het responspercentage in de geselecteerde patiëntengroep kunnen toenemen en wordt eventuele toxiciteit van een niet-effectieve behandeling bij de andere patiënten voorkomen. Hopelijk zal de ontwikkeling van op het individu toegespitste behandelingen, gebaseerd op het

moleculaire tumorprofiel van de betreffende patiënt, uiteindelijk leiden tot een toename van de overleving onder kankerpatiënten.

References

1. Kashkar H. X-linked inhibitor of apoptosis: a chemoresistance factor or a hollow promise. Clin Cancer Res 2010;16:4496-502
2. Stadel D, Mohr A, Ref C, et al. TRAIL-induced apoptosis is preferentially mediated via TRAIL receptor 1 in pancreatic carcinoma cells and profoundly enhanced by XIAP inhibitors. Clin Cancer Res 2010;16:5734-49
3. Brown CJ, Lain S, Verma CS, et al. Awakening guardian angels: drugging the p53 pathway. Nat Rev Cancer 2009;9:862-73
4. Meijer A, Kruyt FA, van der Zee AG, et al. Nutlin-3 preferentially sensitizes wild type p53 expressing cancer cells to DR5-selective rhTRAIL over rhTRAIL. Targeting apoptosis in cancer cells: improving TRAIL death receptor signalling (thesis)
5. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74
6. Vale C, Tierney JF, Stewart LA, et al. Chemoradiotherapy for Cervical Cancer Meta-Analysis Collaboration Reducing uncertainties about the effects of chemoradiotherapy for cervical cancer: a systematic review and meta-analysis of individual patient data from 18 randomized trials. J Clin Oncol 2008;26:5802-12
7. Maduro JH, de Vries EG, Meersma GJ, et al. Targeting pro-apoptotic trail receptors sensitizes HeLa cervical cancer cells to irradiation-induced apoptosis. Int J Radiat Oncol Biol Phys 2008;72:543-52
8. Heijink DM, Jalving M, Oosterhuis D, et al. TNF-related apoptosis-inducing ligand cooperates with NSAIDs via activated Wnt signalling in (pre)malignant colon cells. J Pathol 2011;223:378-89

Dankwoord

Fulltime onderzoek doen is prachtig. Ik had al zo'n vermoeden toen ik aan mijn promotietraject begon en kan dat nu alleen maar bevestigen. Een proefschrift afronden naast de specialistenopleiding is lastig, maar het is gelukt! Om mijn studies goed te laten verlopen, was nauwe samenwerking met tal van mensen een vereiste. Ik wil graag iedereen die bij mijn onderzoek betrokken was hartelijk bedanken, waarvan enkele personen in het bijzonder:

Mijn promotoren prof. dr. J.A. Gietema, prof. dr. E.G.E. de Vries en prof. dr. S. de Jong. Beste Jourik, Liesbeth en Steven. Ik wil jullie heel hartelijk danken voor de mogelijkheid die jullie me hebben gegeven binnen jullie groep onderzoek te doen. Het voelde als een voorrecht in zo'n motiverende, bruisende omgeving te werken.

Jourik, heel veel dank voor alles. Onder andere voor wat je me hebt geleerd over –de specifieke– fase-1 patiëntenzorg. Daarnaast confronteerde je nauwkeurigheid bij het uitoefenen van de wetenschap in z'n volle omvang me regelmatig met mijn eigen kort-door-de-bocht-zijn. Het heeft mijn denken genuanceerd en de manuscripten werden er altijd beter van.

Liesbeth, vele malen kwam ik met een, in mijn ogen, probleem bij je binnen. Er was altijd wel even tijd. Net zo vaak stond ik binnen no-time juichend weer buiten met een richtinggevende aanwijzing of oplossing. Het is slechts een van de aspecten die je snelheid en overzicht kenmerken. Je bent een enorme drive voor mijn promotie geweest. Bedankt voor alles.

Steven, je onuitputtelijke biologische kennis deed me regelmatig duizelen, maar ik heb er erg veel van geleerd. Terwijl ik af en toe voorzichtig over het translationele muurtje richting laboratorium keek, vertaalde jij moeiteloos de laboratoriumresultaten naar de kliniek. Dat maakt het voor een dokter prettig samenwerken met jou. Heel erg veel dank daarvoor.

De leden van de leescommissie, prof. dr. F.A.E. Kruyt, prof. dr. S. Sleijfer en prof. dr. J.G.W. Kosterink wil ik hartelijk danken voor de beoordeling van mijn proefschrift.

Heel veel dank ben ik verschuldigd aan alle patiënten die hebben meegewerkt aan onze onderzoeken. In waarschijnlijk de moeilijkste fase van hun leven gaven ze toestemming voor deelname in een van de studies en zo hebben ze bijgedragen de kennis over kankerbehandelingen verder te vergroten.

Fase-1 studies in Groningen doe je samen met Rotterdam. Prof. dr. J. Verweij, prof. dr. S. Sleijfer, dr. F.A.L.M. Eskens, dr. W.J. Loos, beste Jaap, Stefan, Ferry en Walter, dank voor de prettige samenwerking.

Dr. C.H. Mom, beste Stijn. Mijn promotietraject begon met het overnemen van jouw studies. Het begin was daardoor flitsend, ik sprong op een rijdende trein. Heel erg bedankt daarvoor.

Beste Martine (den Hollander). Ik heb mijn studies vol vertrouwen aan je over kunnen dragen. Ik wens je heel veel succes met het afronden van jouw boekje.

Beste Gerry (Sieling). Zonder jouw ondersteuning bij (onder andere) het kinetieken was het moeilijk geworden alle buisjes bloed op tijd af te nemen, laat staan op vakantie te kunnen. Heel erg bedankt voor alles.

Alle co-auteurs, bedankt voor de altijd goed verlopende, efficiënte samenwerking.

Dr. E.C.F. Dijkers, beste Eli. Bedankt voor de prettige samenwerking tijdens ons mapatumumab-imaging project. Het was een uitdaging de vele data overzichtelijk te duiden en op te schrijven, maar het resultaat mag er zijn!

Dr. J.J. Koornstra, beste Jan Jacob. Toen ik als student bij jou aan mijn afstudeeronderzoek begon, had ik niet kunnen bedenken dat het een hoofdstuk van mijn proefschrift zou worden. Toch had je me al heel snel enthousiast gemaakt voor wetenschap en dat was uiteindelijk een van mijn redenen om aan een promotie te beginnen. Dankjewel daarvoor.

Hoe afhankelijk het goed verlopen van een studie is van de goodwill van veel verschillende partijen tegelijkertijd wordt denk ik het beste weerspiegeld door alle betrokkenen die hebben meegewerkt aan de totstandkoming van hoofdstuk 7 te benoemen: medewerkers van de poli Medische Oncologie, van "het priklab", de apotheek, verpleegafdeling D2VA, het dagcentrum, het nucleaire laboratorium en de afdeling nucleaire geneeskunde. Door jullie inzet hebben we al het kostbare patiëntenmateriaal adequaat kunnen verzamelen en analyseren. Allemaal heel erg bedankt.

Nynke Zwart, bedankt voor je hulp bij de immunohistochemie en DNA-isolatie.

Hans ter Veen, ik ben je veel dank verschuldigd. Vooral voor het -op je thuispc!- op- en uitzoeken van alle mapatumumabscans die min of meer zoek waren geraakt tijdens de verbouwing van de afdeling Nucleaire Geneeskunde.

Dr. J.R. de Jong, beste Johan. Dankjewel voor je rekenwerk aan de dosimetriedata.

Dr. A.H. Brouwers, beste Adrienne. Dank dat je ondanks je drukke werkzaamheden altijd op korte termijn tijd had om de mapatumumabscans samen te bekijken.

Gretha Beuker en Bianca Smit, veel dank voor al jullie ondersteuning de afgelopen jaren.

Alle (ex-)bewoners van kamer Y3.187, heel veel dank voor alle gezelligheid door de jaren heen. Ik ben er hardcore koffieverslaafde geworden en kan meer negerzoenen achter elkaar eten dan ik ooit voor mogelijk heb gehouden (bedankt Renske!).

Lieve tante Gré, heel erg bedankt voor het ontwerpen van de omslagafbeelding. Het is prachtig geworden!

Lieve vrienden, dank voor alle gezelligheid en vriendschap van de afgelopen jaren. Het is fijn te weten dat het goed zit, ook als er een periode erg weinig tijd is om elkaar te zien.

Lieve Geanne en Rian, fijn dat jullie mijn paranimfen willen zijn!

Lieve Jan, broertje, bedankt voor de gezellige avondjes samen in het mooie Groningen.

Lieve pa en ma. Het is geweldig zulke ouders te hebben! Bedankt voor jullie altijd aanwezige interesse in mijn doen en laten, de relativerende woorden en voor alles wat jullie me hebben meegegeven.

Lieve Jessica. Het is fantastisch om een vrouw zoals jij naast me te hebben. Dank voor alle ruimte die je me gaf om dit proefschrift af te ronden. Ik verheug me op alles in de toekomst samen met jou! Ik heb je intens lief.

Corina